ANALYSIS OF THE COURTSHIP BEHAVIOR OF *HELIOTHIS SUBFLEXA* (GUENEE) (LEPIDOPTERA: NOCTUIDAE) AND ITS ASSOCIATED HYBRID

By

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Crosses between Heliothis subflexa (Guenée) females and tobacco budworm (TBW), H. virescens (F.) males produce fertile females and sterile male progeny. Hybrid females backcrossed with H. virescens perpetuate the sterility factor in male progeny. The release of backcross insects has been proposed as a useful strategy for managing TBW populations. To be effective in control of TBW, however, the backcross insects must compete effectively with feral TBW insects. Therefore, in this study behavioral and chemical interactions between parental species and backcross insects are described.

Periodicity of mating resulted from a circadian release of pheromone by H. subflexa, H. virescens, and backcross insects, and a correlated response rhythm in males. The

courtship behavior sequence of H. subflexa males included oriented flight, landing, antennation, attempted copulation, and mating. Backcross males did not antennate, but they fully displayed their hairpencils as did H. virescens males. Behaviors that increased the probability of successful matings were identified as an acquiescent female, antennating by H. subflexa males, full hairpencil display (BC males), and the male's ability to clasp the female genitalia.

Wind tunnel studies indicated that (Z)-9-hexadecenal and (Z)-11-hexadecenal were essential for eliciting H. subflexa male courtship behavior. (Z)-7, (Z)-9, and (Z)-11-hexadecen-1-ol acetates and (Z)-11-hexadecen-1-ol induced subtle behavioral effects in H. subflexa males for landing and in the time that they remained on the source. In field studies, addition of (Z)-11-hexadecen-1-ol (emission 2-3%) to the synthetic mixture increased male captures in sticky and bucket traps.

Pheromone components of backcross females were identical to those found in the pheromone gland of H. virescens females. Data from wind tunnel studies suggest that early backcross ($<BC_6$) male generations may not be as competitive, for mating, as males from later generations ($>BC_6$). Females that mated with BC_{15} males received a greater number of spermatophores than females that mated with parental males.

Removal of the gonads from either male or female H. subflexa demonstrated that gonads had no apparent influence either on the calling behavior and pheromone release of females or on the male's behavioral response to sex pheromone.

CHAPTER 1

INTRODUCTION AND RESEARCH AIMS

Introduction

The tobacco budworm (TBW), Heliothis virescens (F.) is one of the main pests of economically important crops in the USA and countries of the American continents. Annual crop losses by TBW and costs of control are estimated at several million dollars (Knipling & Stadelbacher 1983). In addition, to the actual costs of TBW control it is difficult to place a monetary value on the negative effects on the environment caused by current practices used to protect cultivars (Knipling & Stadelbacher 1983, Metcalf 1988). These problems will only increase in the future because continuous application of insecticide has resulted in H. virescens developing almost complete multiple resistance to the principal classes of insecticides (Brattsten et al. 1986).

During the last decade, the development of alternative methods that minimize environmental hazards while managing Heliothis problems has been given emphasis (Knipling & Stadelbacher 1983). One possibility for the control of H. virescens is by the release of sterile moths. This

technique has been considered as an alternative since 1972 when Laster (1972) reported hybrid sterility in male progeny from interspecific crosses between H. subflexa (Guenée) females and H. virescens males. Although the female progeny were fertile when they were backcrossed with H. virescens males, the male progeny were found to be sterile. Release of these backcross (BC) insects has been proposed as a technique for managing populations of the TBW (Laster 1972, Makela & Huettel 1979, Martin et al. 1984, Laster et al. 1988).

Behavioral and pheromonal chemical studies of H. virescens have been extensive (Roelofs et al. 1974, Tumlinson et al. 1975, Tingle et al. 1978, Klun et al. 1979, Klun et al. 1980, Teal 1981, Pope et al. 1982, Vetter & Baker 1983, Teal et al. 1986). On the other hand, H. subflexa is an innocuous insect that feeds exclusively on groundcherry fruits, Physalis spp. (Brazzel et al. 1953). Heliothis subflexa is of such little importance that it has no common name. Consequently, limited data are available on its pheromonal communication system.

Heliothis subflexa pheromone was identified by Teal (1981), and by Klun et al. (1982). However, when the synthetic blend was field tested in sticky traps, significantly fewer captures of conspecific males relative to captures in similar traps baited with virgin females were observed (Teal 1981). These results indicated that

chemicals which regulate close-range behaviors were absent, masked or inhibited in the synthetic pheromone blend.

Limited analyses of relationships between BC insects with parental species have been documented in laboratory studies. Karpenko & Proshold (1977) studied fertility and mating performance of hybrid and backcross insects from the reciprocal interspecific cross (H. virescens ♀ x H. subflexa ♂). The hybrid males and the backcross male progeny were sterile for three generations. However, by continued backcrossing of female hybrids to H. virescens or H. subflexa males, fertility was restored in backcross males after the third generation. In addition, Pair et al. (1977) analyzed mating dynamics of backcross insects. Nevertheless, the interpretation of the mating behaviors was limited because the experiments were carried out with insects confined in plastic cages where it was most difficult to observe the courtship behavior sequence.

Other studies of backcross insects have emphasized the genetic and physiological basis of hybrid sterility (Proshold & LaChance 1974, Goodpasture et al. 1980, LaChance 1984, Miller 1987), the identification of the pheromone chemicals emitted by BC females (Klun et al. 1982) or the performance of backcross insects under field conditions (Carpenter et al. 1978, Laster et al. 1978, Tingle et al. 1978, Raulston et al. 1979, Proshold 1983, Proshold et al. 1983, Martin et al. 1984). However, the courtship behavior

of backcross insects or H. subflexa has not been studied in detail.

Objectives

Basic behavioral studies of BC and H. subflexa insects would contribute to an understanding of the mechanisms operative in sexual communication among these species and could eventually lead to the development of an alternative control strategy for the tobacco budworm. Therefore, the goals of this study were as follows:

1. to describe the courtship behavior of H. subflexa and selected backcross generations under wind tunnel conditions;
2. to determine in laboratory and field experiments the relationship between pheromone components and evoked courtship behaviors in H. subflexa males;
3. to document the behavioral and chemical interactions between backcross and the parental species under laboratory conditions;
4. to assess possible influences that the gonad endocrine system may have on the courtship behavior of H. subflexa.

CHAPTER 2

REVIEW OF LITERATURE

Insects

Taxonomy. Heliothis virescens (F.) and H. subflexa (Guenée) are members of the subfamily Heliothidinae (Lepidoptera: Noctuidae). Both species are almost morphologically indistinguishable from each other. At times, this resemblance has caused confusion in classification. H. subflexa (Hs) was described originally by M. Achille Guenée in 1852 and conserved its species status until 1938, when McDunnough's Check List of Lepidoptera (cited by McElvare 1941) classified H. subflexa as a geographical race of H. virescens. However, McElvare (1941) indicated that structural differences in the larva, genitalia and the wing banding between these two species permitted restoration of H. subflexa to species status.

Brazzel et al. (1953) noted that differences in the female genitalia are variable and are not reliable for identifying females of the two species. In addition, they established that only the H. subflexa male has immaculate hind wings, while H. subflexa female hind wings have a dark band like the hind wings of H. virescens. Brazzel et al. (1953) were in agreement with most of the description of H.

virescens male genitalia by Mc Elvare (1941), but they noted that H. virescens hairpencils are also present in the posterior end of the male eighth abdominal segment. Thus, position of the hairpencils, male immaculate hind wings and host specificity are the main characteristics for distinguishing H. subflexa from its sibling species H. virescens.

Heliothis virescens (F.) was described originally by Fabricius in 1781 (Todd 1978). This species has been described also as Noctua virescens (F.), Phalaena rhesia Smith, Xanthia prasina Walker, and H. spectanda Strecker, and placed in the genera Aspila, Chloridea, Noctua, and Phlaena by several authors (Todd 1978). The ESA committee on "Common Names of Insects" (Sutherland 1978) has approved the common name of tobacco budworm (TBW) for H. virescens.

Biological aspects. Heliothis subflexa larvae feed only on groundcherry, Physalis spp. (Brazzel et al. 1953). Saray & Loya-Ramirez (1978) described the life cycle of this insect on Physalis ixocarpa Brot., a commercial cultivar in Mexico. They report that the female oviposits on the lower epidermis of leaves, stem and flowers of Physalis spp. Mitchell & Heath (1987) have found allelochemicals in leaves and stems of P. angulata that influence oviposition behavior of H. subflexa females. Upon hatching (three to five days), the larva feeds initially on leaves, but soon it tunnels into small groundcherry fruits where it remains (two to

three weeks). One larva may feed on several fruits ("tomatillos"). After the fourth molt, the larva falls to the ground and burrows into the soil. Several days later (7 to 14), the adult emerges to initiate a new cycle.

When uncontrolled, the larva may destroy up to 90 percent of the P. ixocarpa fruits. Lannate (90 percent, 1g/l/ha) and methyl parathion (50 percent, 3cc/l/ha) are the insecticides commonly used for H. subflexa control (Saray & Loya-Ramirez 1978).

Heliothis virescens has a broader host range and can develop on cultivated hosts such as cotton, Gossypium hirsutum L.; tobacco, Nicotiana tabaccum L.; soybean, Glycine max (L.); corn, Zea mays L.; tomato, Lycopersicon esculentum Miller; and on several wild hosts including: crimson clover, Trifolium incarnatum L.; geranium, Geranium carolinianum L.; persian clover, Trifolium resupinatum L.; and Desmodium spp. (Brazzel et al. 1953, Laster 1982, Avison 1988).

The life history of the TBW is similar to that reported for H. subflexa insects. Eggs are laid singly, on terminal or axillar buds of hosts, and hatch in 3 to 10 days. Larval feeding is completed in two to seven weeks depending on the host and temperature (Brazzel et al. 1953, Kogan et al. 1978). The prepupa exits the plant and burrows into the soil where it remains as a pupa for 12 to 32 days. In this stage the TBW can enter diapause (Kogan et al. 1978). The

oviposition period can vary from 4 to 27 days (Brazzel et al. 1953). The biological characteristics of H. virescens such as mobility, fecundity, and a particular capacity to develop resistance to a broad range of insecticides combine to permit outbreaks of TBW which can lead to significant losses of crops (Wolfenbarger 1973, Kogan et al. 1978).

Hybrid and backcross insects. Laster (1972) has described the life history of hybrids and backcross insects under laboratory conditions. In these interspecific matings, insects resulting from crosses between H. subflexa females and H. virescens males (or their reciprocals) comprise the F_1 generation, and they are named as hybrids. Progeny of the fertile F_1 females crossed with parental males constitute the backcross insects (BC) (Fig. 2-1).

Hybrids and backcross insects grow normally on artificial diet and, based on Laster (1972), their life cycle is as follows: both sexes required an average of 16 days in the larval stage. Male F_1 larvae showed, on average, earlier pupation (2.6 days before females) than F_1 female larvae. Female pupae were heavier than male pupae (442 and 308 mg, respectively). Most of the females entered diapause (some for 2 years); therefore, female emergence was variable. Adult males emerged between 10 and 18 days.

The hybrid colony produced at the Gainesville laboratory (26 ± 3°C, 14:10 L:D cycle) and used for this study gave the following results: larval stage for males was

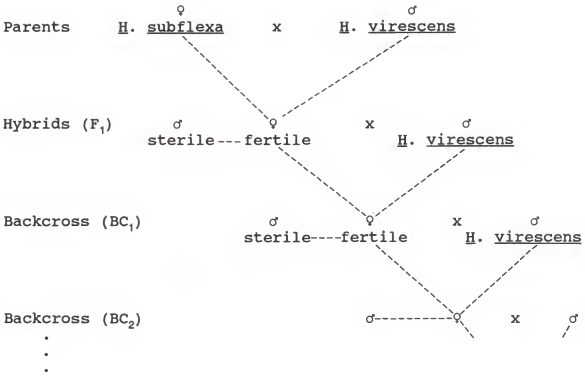

Fig. 2-1. Diagram of parental relationships among H. virescens, H. subflexa, hybrids and backcross insects.

completed from 14 to 21 days, while females that did not enter diapause (50 percent) emerged within 16 to 24 days. Some of the remaining female pupae emerged 3, 6, 14, or 18 months later. After two years several diapausing pupae were still alive. Average weights of female and male F_1 pupae were 485 and 326 mg, respectively, in agreement with Laster (1972). During backcrossing the earlier emergence of males was lost and the weights of both sexes were similar.

Host acceptance and development of hybrids and backcross progeny on several plant species was studied by Laster et al. (1982). These studies demonstrated that F_1 insects were able to complete development on cotton, soybean and groundcherry. Feeding behavior of BC insects (> BC_6) was similar to their recurrent parent (H. virescens), apparently due to the increased proportion of TBW genes in the BC individuals (Laster et al. 1982). Consequently, later BC generations (> BC_3) developed normally on cotton, corn, soybean, and wild geranium, but they did not survive on groundcherry plants. Host preference studies by Avison (1988) showed that hybrid larvae favored grouncherry plants over cotton, but under no-choice situations they could grow on cotton. Host preference by BC_3 larvae was similar to that exhibited by TBW larvae.

Behavioral Studies

In behavioral studies the bioassay frequently is the basis for elaborating hypothesis about the behavioral

mechanisms operative in chemical communication systems. Wolfson (1988) has defined bioassay as ..."any assay in which a living organism is permitted to declare whether a specific variable makes a difference as to how the individual fares" (p. 1951). Obviously, the complexity of the interaction between the individual and the surroundings can not be reproduced totally in the bioassay. Therefore, the intrinsic limitations of the bioassay should be considered when planning behavioral tests (Wolfson 1988). Martin & Bateson (1986) discuss the general steps involved in studying behavior, while Baker & Cardé (1984) and Wolfson (1988) mention specific considerations for conducting bioassays in insects. Baker & Cardé (1984) emphasize that insect responses made in-flight integrate internal and external information. Therefore, bioassays utilizing flight in wind are probably the most discriminating in pheromone research.

Mating Behavior in Noctuidae

There is extensive literature on the mating behavior of noctuid species; however, only the mating aspects applied to this study will be outlined. Usually, the courtship behavior begins when the female extrudes and protrudes her abdomen to expose the pheromone gland to the environment.

Conner & Best (1988) discuss the dynamics of the postural changes of females associated with pheromone release. Wing vibration can accompany this calling

behavior, as in H. zea (Boddie) (Agee 1969), Leucania separata W. and Mamestra brassicae W. (Hirai 1977), but not in H. virescens and H. subflexa (Teal 1981), Spodoptera littoralis Boisd. (Ellis & Brimacombe 1980), and Anticarsia gemmatalis (Hübner) (Leppla et al. 1987). Dragging of the ovipositor against the substrate is common in the above mentioned species. Teal (1981) indicates that these scent marks may contribute to eliciting male courtship behaviors.

Noctuid females may display the "calling posture" in the first or second night after emergence. The exhibition of this behavior, often accompanied by pheromone release, is under control of endogenous oscillators entrained mainly by the photoperiod (Saunders 1982). The endogenous nature of a rhythm usually is revealed when the individual is transferred from a light/dark-cycle (LD) into continuous dark (D) or continuous light (L) (Saunders 1982).

Others factors, such as temperature, humidity and physiological status can modify the expression of these circadian rhythms (Saunders 1982). In moths, these oscillations provide a temporal organization for physiological and behavioral activities (i.e., calling, male searching behaviors), allowing synchrony in intraspecific chemical communication (Saunders 1982, Raina & Menn 1987).

Daily cycles of pheromone release have been documented in Trichoplusia ni (Hübner) (Sower et al. 1970), and H. armigera (Kou & Chow 1987). Daily rhythms of calling

behavior for other noctuid species have been described, but the experiments were conducted with the insects maintained under cyclical rather than constant conditions (Saunders 1982).

Under natural conditions, noctuid species call during the scotophase. Heliothis zea calls between 1 to 10 h, with a peak at 4 h; H. virescens between 3 to 9 h, with a peak at 6 h (Tingle et al. 1978, Teal 1981); H. subflexa between 3 to 6 h, with a peak at 5 h (Heath et al. 1988); and H. armigera between 1 to 4 h, with a peak at 3 h (Kou & Chow 1987).

A synopsis of courtship behaviors observed in moths (Agee 1969, Ellis & Brimacombe 1980, Teal 1981, Leppla et al. 1987) include adoption of calling posture by the female; male activation with antenna raised; wing vibration; initiation of male flight either random or oriented within the plume; landing either parallel to or below the female body, although T. ni males align above and slightly in front of the female. On source males make full extrusion of claspers and hairpencils and at this point they may antennate the female body and attempt copulation with bending of the male abdomen toward the female for clasping her genitalia. If proper contact is made, copulation follows. Typically, the engaged moths move to a position with heads directed away.

Ellis & Brimacombe (1980) noted that reactions of males (activation) to pheromone perception also were observed when S. littoralis males exhibited feeding behavior, so these reactions may signify general arousal of the insect to environmental stimuli.

A comparison of the behaviors between successful and unsuccessful males indicates that acquiescence of the female, accurate parallel movement of the male toward the female, and the male's ability to clasp the female genitalia are requisites for successful matings. The role of the hairpencils is variable. In S. littoralis (Ellis & Brimacombe 1980) and H. virescens (Teal 1981) a hovering display is strongly related to a successful mating, while for A. gemmatalis (Leppla et al. 1987), male antennation and thrust were the behaviors decisive for female acceptance.

Although the mating behavior sequence of these moths is relatively stereotyped, a certain amount of flexibility is present. Thus, as in any population, some individuals may mate successfully without performing all the behaviors in the sequence. Conversely, others may execute all steps in the courtship behavior and still be unable to mate.

Chemical Composition of Pheromones in Heliothis spp.

Because of the economic importance of Heliothis species (Knipling & Stadelbacher 1983), their chemical communication systems have attracted considerable attention from chemists and entomologists (Tamaki 1985). The pheromone blend of the

studied Heliothis species contains alcohols, acetates, and aldehydes of aliphatic straight-chain compounds (Table 2-2).

Data shown in Table 2-2 indicate that (Z)-9-hexadecenal, (Z)-11-hexadecenal, hexadecanal, and (Z)-11-hexadecen-1-ol are common pheromonal components in the sex pheromone of Heliothis species for which the sex pheromones have been identified. This common chemical background provides us with interesting relationships between the chemical structures and biochemical pathways with the taxonomical position of these species.

Differences in composition ratio and presence of trace chemicals may contribute to specificity in a particular signaling system. For example, acetates are found only in H. subflexa (Teal 1981), and probably they are essential to pheromonal activity and distinctive in this species. Similarly, (Z)-9-tetradecenal and tetradecanal have been implicated in the elicitation of upwind flight in TBW males (Vetter & Baker 1983). Sometimes the same compound may evoke a different behavioral response in different species. Thus, (Z)-11-hexadecen-1-ol is critical for eliciting a maximal response in H. phloxiphaga (Raina et al. 1986), but it causes a significant decrease in H. zea (Teal et al. 1984) and H. virescens male response (Pope et al. 1982).

Conversely, some compounds may play similar roles in the behavioral repertoire of the studied Heliothis species. Vetter & Baker (1983) found that deletion of (Z)-11-hexa-

Table 2-2. Percent composition of washes from abdominal tips of *Heliothis* spp.

Chemical	Species Ha[a]	Hs[b]	Hv[c]	Hz[d]	Hp[e]
(Z)-9-tetradecenal	--	--	2.0	--	--
Tetradecanal	--	--	1.6	--	--
(Z)-7-hexadecenal	--	--	1.0	--	--
(Z)-9-hexadecenal	3.2	19.8	1.3	0.9	0.4
(Z)-11-hexadecenal	96.1	30.0	81.4	66.9	86.3
Hexadecanal	--	5.4	9.5	2.8	7.1
(Z)-11-hexadecen-1-ol	0.7	12.2	3.2	27.9	6.2
(Z)-7-hexadecen-1-ol ac	--	1.6	--	--	--
(Z)-9-hexadecen-1-ol ac	--	4.3	--	--	--
(Z)-11-hexadecen-1-ol ac	--	12.3	--	--	--

[a] H. armigera, Kehat et al. 1980.
[b] H. subflexa, Teal 1981.
[c] H. virescens, Klun et al. 1980.
[d] H. zea, Teal et al. 1984.
[e] H. phloxiphaga, Raina et al. 1986.

decenal from the pheromonal blend, resulted in no TBW male response. This aldehyde is the main compound in all studied Heliothis sex pheromones, and probably it is essential for initiating male behaviors in these species.

The identification of courtship behaviors associated with pheromonal components may contribute to an understanding of the behavioral mechanisms involved in the chemical communication systems of Heliothis species. Eventually, such basic behavioral studies will serve as the foundation for more practical control methodologies using insect pheromones (Palaniswamy & Underhill 1988, Cardé 1988).

Hybridization Studies

Since the discovery of inherited backcross male sterility, that results from the crossing of H. subflexa females with H. virescens males, there has been considerable interest in the potential of using the sterile males as a method of controlling TBW populations (Laster 1972, Makela & Huettel 1979, Laster et al. 1988). If BC insects are competitively identical to wild TBW moths for matings, the production of sterile males would be self-perpetuating with the possibility of reducing local TBW populations (Makela & Huettel 1979).

Evaluation of the competitiveness of BC insects for mating with H. virescens moths is needed to predict the sexual performance of the released backcross under field

conditions. Initial laboratory studies by Proshold & LaChance (1974) showed that H. virescens males mated readily with BC females. However, H. virescens females which mated with BC males failed to lay a normal number of eggs and remated readily. Later, Proshold et al. (1975) explained that these effects were caused by the inability of BC males to transfer eupyrene sperm to the spermatheca of a female.

Hybrid females backcrossed to H. virescens always produce sterile male progeny (Laster 1972). However, when hybrid (reciprocal cross) females were backcrossed with H. subflexa males, fertile BC males existed by the third generation (BC_3); Thus, Karpenko & Proshold (1977) hypothesized that maternal effects (chromosomal and extrachromosomal) could explain the persistence of male BC sterility. In the same study females mated with BC males received a greater number of spermatophores than females mated with normal males.

Pair et al. (1977) found that BC females behaved similarly to H. virescens females in synchrony (calling time) and mating. Although females crossed with BC males showed increased coital response in comparison with females crossed with H. virescens males, they predicted that BC females should be able to intermate with natural TBW populations.

Field tests using sex pheromone traps baited with BC and normal TBW (laboratory-reared) insects indicated that BC

females could compete with H. virescens females in attracting native TBW males (Laster et al. 1978). Also, Carpenter et al. (1979), using mating tables in a tobacco field, proved that BC females were competitive with TBW females.

Raulston et al. (1979) made field studies to assess mating interactions between a natural population of TBW and released BC insects. Their results indicated that BC females attracted native males, but BC males were not competitive for mating with wild TBW females. They stressed the necessity of using parental cultures from the target area, for "manufacturing" BC cultures. Thus, insects with a similar genetic background would minimize differences in mating and feeding habits between released and native moths.

An extensive release of BC population on St. Croix, U.S. Virgin Islands (Proshold 1983, Proshold et al. 1983) revealed several favorable facts: BC field-reared (BCF) insects increased their frequencies after each release, BC moths dispersed homogeneously throughout the island, and they survived the dry season. Also, it was observed that some type of selection was operating against BCF insects because their populations declined with time. These researchers recommended that BC to TBW ratios of 30:1 or higher would be necessary for controlling H. virescens.

Martin et al. (1984) released BC moths in Puerto Rico. They observed that released females mated readily with feral males, but the percent of BC males mating with natural

females was low. The above mentioned study suggests that further behavioral assessment is needed before the sterile male trait can become a valuable tool in TBW control practices.

Role of Ovaries and Testes on Courtship Behavior

Females of lepidopterous species produce sex pheromones which attract males and initiate courtship behavior. Females which are short-lived as adults (i.e., Hyalophora cecropia (L.), Galleria mellonella (L.)) must attract and mate in a brief time span. Females of these species emerge with almost a full complement of eggs, and soon (1-2) days) thereafter begin to exhibit calling behavior.

Work on Lymantria dispar (L.) by Hollander & Yin (1982) has shown that calling behavior and pheromone release are two separate physiological events under different regulatory mechanisms. Allatectomy in L. dispar did not stop female calling behavior or pheromone release. However, brain removal or brain disconnection prevented pheromone release from calling females (Hollander & Yin 1985). Removal of corpora allata had no significant effect on calling of Manduca sexta (L.) (Itagaky & Conner 1986). A neural output from the brain and/or the subesophageal ganglion was suggested as the control mechanism of these behaviors.

In addition to the effects of cephalic endocrine structures on regulation of calling and pheromone release, ovaries have been examined as possible modulators of these physiological process with variable results. Barth & Lester

(1973) reported that ovaries are important for the exhibition of calling behavior and pheromone release in the cockroach. On the other hand, ovariectomy in L. dispar did not interfere with either calling or pheromone release (Hollander & Yin 1985).

There are a few studies on the effect of male castration on courtship behavior performance. Castrated H. cecropia pupae formed apparently normal males that mated and produced sterile spermatophores. Females mated with these sterile males failed to switch to an oviposition pattern (Riddiford & Ashenhurst 1973). Similarly, castration of M. sexta males did not incapacitate them for mating, but females mated to castrated males showed only a transient cessation of calling. Females mated to normal males did not call a second time (Sasaky & Riddiford 1984). It would be interesting to know if the extirpation of gonads in H. subflexa causes some effects on the courtship behavior of adults.

CHAPTER 3

COURTSHIP BEHAVIOR OF HELIOTHIS SUBFLEXA AND BACKCROSS INSECTS

Introduction

Heliothis virescens (F.) is an endemic species that is among the most damaging pests of agriculture in the United States and Mexico. It is highly mobile and attacks a wide range of host plants. The severity of the Heliothis problems in these areas is in large measure the consequence of our greatly altered agroecosystem (Metcalf 1988). Considering its economic importance, the need for acceptable alternatives to chemical insecticides, and for minimizing the crop losses due to this pest, the phenomenon of inherited hybrid male sterility between H. virescens and H. subflexa (Guenée) has been the subject of considerable investigation.

Laster (1972) reported hybrid sterility in F_1 male progeny from interspecific crosses between H. subflexa females and H. virescens males. The male progeny were completely sterile when mated to H. virescens or H. subflexa females. The F_1 hybrid males also were sterile when crossed with the F_1 females. Viable fertilizations occurred when the hybrid females were backcrossed with H. virescens males.

On the basis of these effects, Laster (1972) proposed that the release of hybrid male progeny would be useful in applying the sterile male method for managing H. virescens. If the potentially disruptive effects on reproduction can be accomplished under natural conditions, this would provide control of H. virescens because of the persistence of sterility in successive generations.

One initial step in predicting the behavior of sterile males under field conditions is to detail the behavioral repertoire of the parental species and selected backcross generations. Such basic behavioral studies will contribute to an understanding of the mechanisms operative in sexual chemical communication, and may eventually lead to the development of an alternative control strategy for the tobacco budworm.

One of the parental species, H. virescens, has been the subject of numerous chemical and behavioral studies (Roelofs et al. 1974, Tumlinson et al. 1975, Teal et al. 1981, Teal et al. 1986). However, behavioral sequences of H. subflexa and backcross insects involved in the mating process have not been studied in detail. Therefore, the present experiments were carried out to describe the courtship behavior sequence of H. subflexa and selected backcross insects under laboratory conditions.

Materials and Methods

Insects. The culture of H. subflexa originated from larvae collected from groundcherry fruits Physalis angulata near Gainesville, FL, and it has been reared in the laboratory since 1983, with feral males added to the mating stock every fall (Mitchell et al. 1988). Larvae were reared according to methods described by Mitchell et al. (1988). Pupae were sexed and transferred to separate environmental chambers in which the light:dark regime was set at 14:10 hours, with lights-off at 3:00 PM, and temperature regulated at 26 ± 2°C. When the adults emerged they were held in their respective chambers either in a 3.8 liter carton (30/carton) or a plexiglass cage (25x25x25 cm) until tested. H. virescens pupae were received from Oxford, NC, on a weekly basis. Sexing and handling of H. virescens were essentially the same as for H. subflexa. All insects were supplied with a plastic cup containing a cotton ball saturated with 4% honey-sugar-water solution.

The hybrid culture was derived from H. subflexa females x H. virescens males, with the female progeny backcrossed to H. virescens. Backcross larvae were reared individually on pinto bean diet in 30 ml plastic cups. Pupae were sexed and held in separate environmental chambers. Moths were collected daily and transferred to new cages so each holding cage contained adult insects of a single age and sex. For maintaining the backcross culture, the BC females were

confined for mating with H. virescens males and the eggs were collected 4-5 days later. Moths from 2 to 5 days old were used once in the experiments and then discarded.

Mating behavior studies. On the basis of preliminary observations, the main calling period (> 60 per cent) of H. subflexa and BC females occurred between 4 to 6, and 6 to 8 hrs, respectively, after lights-off. Fluctuations in responsiveness are the rule in pheromone research and thus the time of assay was first maximized to the period when the insects demonstrated the most intense response to the pheromone. The fifth backcross generation (BC_5) and sixth generation (BC_6) insects were used in experiments for determining courtship behavior sequence. Bioassays were performed in a plexiglass wind tunnel (0.75 m wide, 0.75 m high, 2 m long), through which air was pulled at a constant rate (0.36 m/sec). Male moths were acclimated for 1 h to wind-tunnel conditions (25 ± 2°C, 1.4 lux light level). A virgin calling female (H. subflexa or BC) was gently positioned on a hanging piece of screenwire (2 x 3 cm) placed in the center of the wind tunnel at the upwind end, and then a conspecific male was released at the downwind end within the pheromone plume (approximated plume boundaries delimited by titanium tetrachloride-generated smoke). The total length for which a behavior pattern (i.e., activation, random flight, oriented flight, landing, antennating, attempted copulation, mating, etc.) lasted, over the

specified period of the observation, was calculated by recording the duration and frequency of behavioral events on a portable Epson® PX-8 computer using a time event-recorder program (Mitchell, unpublished data). Males were monitored during a 2 min period and recorded on videotape using a RCA®-TC2000 camera equipped with macro lense and a Panasonic® PV-8500 cassette recorder. The visual record of the behavior was subsequently slowed down for back-up to live observations. The terminology is that of Teal (1981).

Male behavioral data were organized in a first-order Markov matrix and examined to find if the qualitative variables forming the matrix were independent or not. In a first-order matrix, the probability of occurrence for the next event depends only on the immediately preceding behavior. In a random sequence, one behavior can be followed by any other (including itself) with equal probability.

The conditional probability that one behavioral event follows another [the probability that B follows A, given that A has occurred, P(B/A)] is referred to as a transition probability (Martin and Bateson 1986). When the two variables are independent, the frequencies estimated and the observed frequencies should only differ by amounts attributable to random chance. If the two behaviors are not independent, large differences would be expected to occur.

Thus, x^2 will be smaller when the two variables are independent than when they are not independent. A method for deciding on values of x^2 which should lead to acceptance of Ho (Ho: variables are independent) and those which should lead to rejection has been commonly used in behavioral studies (Stevenson & Poole 1976, Fagen & Young 1978, Teal et al. 1981, Burns & Teal 1988). In this procedure Oij = observed frequency in cell (i,j), Eij = expected calculated frequency and $Y = (Oij - Eij)/ (Eij)^{.5}$. If $|Y| > (x^2_{.05}, d.f.)^{.5} \div R$ (repertoire size = 7) (Bishop et al. 1975), then the transition (act i → act j) is occurring at a frequency that differs significantly ($P < 0.05$) from chance prediction. Transitions with standard normal deviations (Stevenson & Poole, 1978; Teal et al. 1981) were calculated and those with probabilities $P < 0.05$ were utilized for constructing a flowchart of courtship behavior of H. subflexa and backcross insects.

Comparisons were made between successful and unsuccessful insects by using the Mann-Whitney U-test. This nonparametric test only requires ordinal measurements and makes no assumptions about the distribution of the data. It is a most useful alternative to the parametric t-test when the t-test's assumptions are not completely valid (Siegel 1956, Conover 1980). Additional comparisons between groups were made using a x^2 2 x 2 test of independence.

Results and Discussion

Precopulatory Behavior

Heliothis subflexa. Observation of female behavior (n = 40) indicated that the majority of female moths (60 percent) began to call within 4 to 6 hrs into the dark phase of the second day after emergence (Fig 3-1a). The typical posture of the calling female began with protrusion of its abdomen and extrusion of its ovipositor. The wings were held in a "V" position and separated from the body, the antennae were erect and oriented forward or laterally. Other females (17 percent) held their wings in contact with the substrate and their abdomens clearly protruded and the pheromone gland extended to its maximum; this was given the term "deep calling state". Females in a deep calling state could be moved easily from one cage to another without disturbing them. In wind tunnel studies, they mated with the first male that arrived regardless of his performance in the sequence. Usually, calling females remained relatively stationary during this behavior; however, some of them moved about in short flights and/or brief walks. Occasionally the pheromone gland was not retracted during these flights or ambulatory periods. Calling behavior of these females stopped immediately when they were moved from one container to another, and they continued shifting from one position to another before resettling.

Females that were not calling during the first session were isolated and observed throughout the course of the following periods. Usually these females began to call during the two following nights; however, a few of them (12 percent) did not call at all even after 5 days. These females (n = 6, 5 day-old) were positioned in the wind tunnel but searching males were unable to find them. Later, these noncalling females were placed on or near to scent marks left by previous calling females. Excited males tried to copulate with them, but the females adopted a "mate-refusal" posture with depressed antennae and wings. If the male persisted in the courtship, non-calling females took flight or dropped from the wind tunnel wall. In comparison females that were calling normally but refused to mate gave a vigorous flick of the wings to the persisting male and then flew away.

The calling behavior of entrained H. subflexa females held 3 days in a 1.3 lux light intensity showed a circadian rhythm (Fig. 3-1a). Females 3-4 days-old began to call earlier and stopped calling later than females 1-2 days-old. Kou & Chow (1987) found that the mean onset calling time of H. armigera (Hübner) changed with the calling age. The mean length of the call increased from 1 h, on day 1, to ca. 2 and 3 hrs on calling days 2 and 3, respectively. Apparently, in these two species older females maximize their reproductive chances by increasing the length of the call.

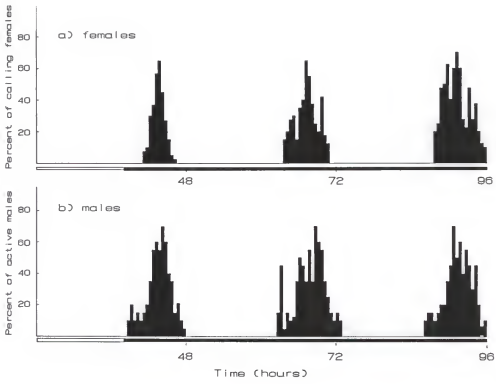

Fig. 3-1. Synchronization of female calling and male flight response periodicities of H. subflexa in wind tunnel conditions (26±2°C, wind speed 0.36m/sec, 1.4 lux). Permanent scotophase indicated by closed time bar.

Daily cycles of pheromone release have been documented in Trichoplusia ni (Hübner) (Sower et al. 1970) and H. armigera (Kou & Chow 1987). Daily rhythms of calling behavior for other noctuids have been described, although the studies were conducted with insects held under cyclical conditions. Under constant conditions an endogenous oscillation controlling a rhythmic activity can "free-run" and reveals the natural periodicity (Saunders 1982).

Some aspects of calling behavior of H. subflexa females were similar to other noctuid moths. Partial elevation of the wings, body, and eversion of the pheromone gland during calling were analogous to calling display exhibited by Plodia interpunctella (Hübner) (Grant 1976) and H. virescens (Teal et al. 1981). However, H. subflexa did not fan their wings while they called as reported for H. zea (Boddie) (Agee 1969) and Anticarsia gemmatalis (Hübner) (Leppla 1987). In some species, e.g. Grapholitha molesta (Busk), wing-fanning helps direct the pheromone to the receiver (Baker & Cardé 1979).

For other insects that do not fan their wings, Conner & Best (1988) proposed that pheromone release is associated with postural changes. They conducted experiments using a thermistor flow probe to measure wind speed around a Spilosoma congrua Walker female and found that the calling posture (similar to H. subflexa) could be described as one that maximizes pheromone release.

Calling posture may influence other aspects of the courtship process as well. A raised-wing posture associated with calling in Hypantria cunea Drury has been shown to enhance the attractiveness of dead and dried females placed in the vicinity of a pheromone source (Hidaka 1973). This visually mediated effect should be considered when working with crude extracts or synthetic material.

Experiments with H. subflexa males were carried out with batches of 20 insects under wind tunnel conditions (26 ± 2°C, wind speed 0.36m/sec, 1.4 lux) to minimize the possibility that one or more hyperactive individuals might stimulate similar activity in other test insects. Flight activity was recorded by visual observation. Figure 3-1b indicates the percent of active males during the recording period. Male activity occurred throughout the entire period, with moderate activity during the first and last third of the scotophase. High mobility (short flight shifting) was synchronized to the onset of female calling. Male activity continued at an elevated level until 7 hr into the scotophase and gradually became more moderate until the majority of males (≈ 80 percent) adopted the resting position around 9 hr into the scotophase. Like female calling behavior, the searching behavior in males persisted under constant dim light (ca. 2 lux) conditions (Fig. 3-1b). A slight spreading of male flight occurred on days 3 and 4 due, probably, to moths getting out of phase.

Intrinsic movement of these males during four nights in the absence of females indicated that male activity recurred in circadian cycles and that it was probably controlled by an internal clock which was entrained by the light-off signal (Saunders, 1982). Thus, periodicity in mating would result from a rhythmic release of pheromone by females, and in this case, by a correlated response rhythm in males. In Holomelina immaculata (Reakirt), an arctiid, the males possessed a circadian response well coordinated with the female calling. However, in H. aurantiaca (Hübner), the males were attracted to females over a much wider time interval (Cardé 1974). In Manduca sexta (L.), females synchronized their calling with the flight time of the males (Sasaki & Riddiford 1984)

The timing of this daily rhythm in both sexes of H. subflexa could be a species-specific characteristic due to behavioral responses to daily changes in the environment and physiological status. Such a coordination of mating activity provides the H. subflexa sexes with a high probability of mate finding with a minimum expense of energy (Raina & Menn 1987), and it might enhance the reproductive isolation from closely related species (Mayr 1970).

Backcross insects. During the daytime backcross females, like other noctuid species, adopted the resting position with the wings overlapping slightly at the apex and the antennae folded beside the folded wings. The calling

period of these females (n = 40), evidenced by the exposure of the pheromone gland, was between 5 and 9 hours after lights-off with a peak period (> 60 percent) from 6 to 8 hours (Fig. 3-2a).

The characteristic calling posture began with the female waving her antennae and vibrating the wings, while extruding and protruding her abdomen to expose the pheromone gland. Wing vibration was manifested only at the beginning of the calling time; wing vibration was not observed during calling when the wings were held above the body in a "V" position. Females alternated bouts of calling with short periods of feeding, walking and flying. Extension of the ovipositor was similar to the ovipositor projection observed in H. virescens females.

Stationary calling females were interrupted often by shifting females causing a discontinuous pattern of calling in both individuals. If the shifting female made physical contact with the calling female both attempted copulation. This "homosexual behavior" (described for H. virescens, Teal 1981) resulted in the females withdrawing their ovipositors and resettling before resumption of the calling behavior. None of the BC females was observed in as profound a calling state as that described for H. subflexa (see page 28). The premating behavior of the backcross females resembled very closely the description of premating behavior of H. virescens females (Teal et al. (1981).

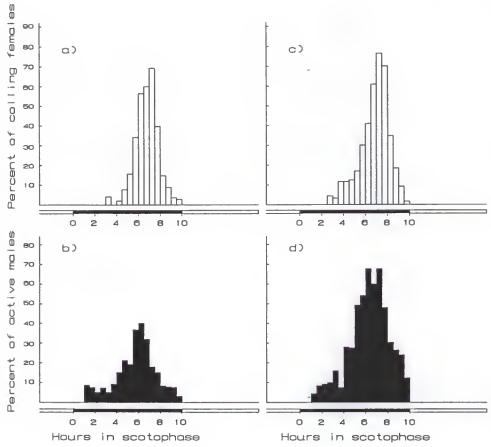

Figure 3-2. Comparison of female calling (open histograms) and male flight (closed histogram) activity between backcross insects and H. virescens. a) and b) backcross insects; c) and d) H. virescens. Scotophase indicated by closed time bar.

The calling interval of the backcross females coincided with the calling period observed in the parental species, H. virescens (Fig. 3-2c). The early part of these interims overlapped the late portion of the H. subflexa calling interval, suggesting that the pheromone release period of the backcross and parental females were not sufficiently distinct to effect a great degree of reproductive isolation. These data are in agreement with results obtained in field experiments conducted by Tingle et al. (1978).

Backcross males began to display searching behavior 4 hours after the beginning of the dark period. Within this period no more than 30 percent of the males were active (Fig. 3-2b). Thus, the majority of them were alert for a few minutes but soon resettled and adopted the resting position in contrast with the high level of activity exhibited by males from H. virescens (Fig. 3-2d). Therefore, in the wind tunnel most backcross males were at rest and began activation only when they detected the pheromone released by females.

Courtship Behavior

Heliothis subflexa. A one step transition table giving frequencies of preceding and following acts in H. subflexa sequence behavior served to detect those involved in male-female communication. In this approach the response of one individual depended on the immediately preceding behavior, a necessary condition for the occurrence of communication.

Behaviors were categorized as follows: activation (Act), random flight (Rf), oriented flight (Of), landing (arrival to source) (La), antennation (An), attempt copulation (Ac), mating (M), and successful mating (Sm). Transitions between established pairs occurring at a frequency that differed significantly ($P < 0.05$) from chance expectation were expressed in the flowchart showed in Fig. 3-3. Probabilities between two adjacent behaviors were estimated from the contingency table according to Bishop et al. (1975).

Courtship behavior commenced when the resting male (antennae and wings folded along the body) began to vibrate his wings and to orient his antennae toward the stimulus source. The male genitalia were partially ($P = 0.70$) or totally protruded from the abdomen ($P = 0.23$). Due to high circadian activity at that time, most of the males were active before or became active when introduced into the wind tunnel. With these males, it was not known whether wing vibration or antennae movement were caused by perception of female pheromone or his intrinsic high level of arousal. However, extrusion of the male's genitalia was a clear signal of activation. When an excited H. subflexa male perceived female pheromone at a level above its behavioral threshold, the male was stimulated to fly. The anemotaxis response triggered by odor response was random ($P = 0.65$) or oriented ($P = 0.30$). Both transitions were not arranged independently (X^2, 0.05); thus, those responses depended to

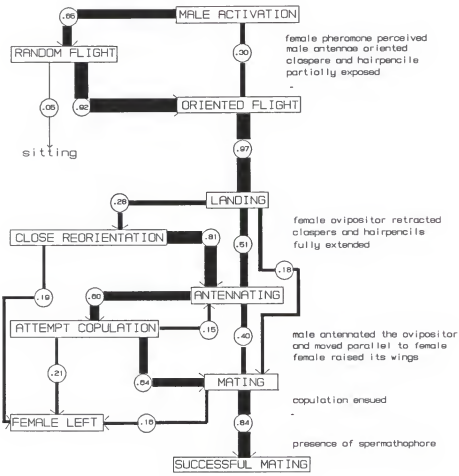

Figure 3-3. Flowchart of the courtship behavior sequence of H. subflexa ($P < 0.05$). Thickness of lines and values in circles indicate the transition probability between two adjacent behaviors.

some extent upon the preceding act of behavior. After a few seconds of random flight, males turned to plume tracking (P = 0.92) and only two of the test insects (n = 43) discontinued the sequence (P = 0.05).

Orientation to the pheromone source was characterized by a zigzag flight alternated with ascending and descending arcs within the pheromone plume. The series of turns decreased in amplitude as the insect came closer to the calling female. Almost all of the oriented males landed (P = 0.97) on the screenwire surface. Landing was either parallel to or below the female's body with full exposure of the male genitalia. At this point, the female retracted the ovipositor and raised her wings. This was followed by antennation of the female's ovipositor (P = 0.51) (if the male approach was overly aggressive, the female moved away, P = 0.19), by reorientation with extension and wiping of his genitalia (P = 0.28), or by immediate copulation (P = 0.18). Antennation consisted of touching the tip of the female abdomen with the antennae for 3-4 seconds. The female responded to antennation by lifting her wings and maintaining the abdomen parallel to the male's abdomen. After antennating, the male bent the posterior part of its abdomen abruptly toward the female's abdominal tip and engaged her genitalia. If the male made proper contact with the female ovipositor (P = 0.40), copulation ensued and the pair moved to a position with heads directed away. Males

that failed to make proper contact tried to copulate again ($P = 0.60$). At this point, some males returned to antennation ($P = 0.15$) or they continued to attempt copulation, with the pair moving parallel until mating ($P = 0.64$). At this stage, some females rejected their mates ($P = 0.21$) by a) curving their abdomen upward so that the male was unable to clasp the female genitalia; b) adopting resting posture with the wings covering the abdomen; or c) flying away and resettling with the net result that these males, after cycling, remained unsuccessful in spite of their persistence.

A successful mating was indicated by the presence of a spermatophore in the bursa copulatrix ($P = 0.84$). In other cases ($P = 0.16$) where pairs apparently were in copula for several minutes, the female abruptly disengaged and moved away from the male. An unidentified but essential factor for male acceptance was missing during this interaction because the males were rejected when they tried to copulate again.

Comparisons of timed behavioral events between successful and unsuccessful males were made using the Mann-Whitney U-test. The z-values corrected for two-tailed probabilities are given in Table 3-1. Unsuccessful males spent significantly more time in random flight than successful males ($z = 2.1$, $P = 0.035$). Although these random flight periods cannot be extrapolated to field

conditions they may indicate that early detection of the pheromone plume would positively influence a male's chance of mating under natural conditions.

There was a significant difference ($z = 3.36$, $P = .001$) between successful and unsuccessful males in the time spent in attempted copulation (Table 3-1). Males that made sexual approaches to females that did not end in mating attempted copulation several times. However, the males failed to clasp the ovipositor. Video analysis showed that the female need only to move away one or two cm to avoid the male. The female rejection behavior involved walking, running or flying away from the male, wing flicking or lifting or curling her abdomen away from the male. Successful males usually moved parallel to the female's body, and then attempted copulation only once or twice before moving to the typical lepidopteran mating position with heads in opposite direction.

There were no significant differences (Mann-Whitney U-test) between successful and unsuccessful males related to mean total duration (sec) oriented flight, landing, and antennating. A x^2, 2 x 2 test of independence (Siegel 1956) was used to examine the percent of successful and unsuccessful males participating in a specific behavior. There were significant differences in percent of plume tracking ($x^2 = 5.5$, $P = 0.018$), landing ($x^2 = 27.1$, $P < 0.0001$) and antennating ($x^2 = 70.3$, $P < 0.0001$) (Table 3-2).

Table 3-1. Comparison of behavior duration (sec) between successful and unsuccessful males of H. subflexa.

Behavior[a]	Successful n	Mean ± S.E.	Unsuccessful n	Mean ± S.E.	Comparison z	P
Rf	21	10.2 ± 1.3	12	18.7 ± 3.4	2.10	.035[b]
Of	27	10.2 ± 1.3	13	12.4 ± 4.2	0.14	.890
La	27	21.8 ± 0.5	11	22.8 ± 3.6	0.21	.833
Ant	23	3.0 ± 0.2	5	2.8 ± 0.4	0.19	.848
Ac	11	2.8 ± 0.4	5	8.4 ± 1.3	3.36	.001[b]

[a] Rf random flight, Of oriented flight, La landing, Ant antennating, Ac attempted copulation.

[b] Means in the same row are significantly different according to a Mann-Whitney U-test.

Flying males that did not orient also failed to land and returned to random flight after a few seconds where they adopted a resting position on the wind tunnel walls. This behavioral response was observed only in a few males, and it probably had limited, if any, influence on overall sexual fitness.

On the other hand, antennating was related to successful mating. Indeed, approaches in which the males antennated the ovipositor ($x^2 = 70.3$, $P < 0.0001$) generally were accompanied by a lifting of the wings by the female and maintenance of the abdomen parallel to the substrate, a position that suggested potentially successful mating. Most of the males that did not antennate the ovipositor evoked in the female the "refusal" response described above, and the females took flight as a result of continuous copulation attempts.

The experiments reported here suggest that there were four major patterns, in addition to a calling female, associated with successful mating: 1) mate location, 2) ovipositor antennation, 3) proper parallel approach to the female body, and 4) a precise copulation attempt. Antennating appeared to be the critical behavioral step where a H. subflexa female recognized a mate. In the closely related species H. virescens, the males did not antennate the female ovipositor (Teal 1981). Nevertheless, they have well developed hairpencils (in comparison with Hs

Table 3-2. Comparison of behaviors recorded in successful and unsuccessful matings of H. subflexa. Values give the percent of courtships in which the behavior was present.

Behavior[a]	Successful	Unsuccessful	Comparison x^2	P
Rf	77	75	.02	.8684
Of	97	87	5.50	.0189[b]
La	97	68	27.15	<.0001[b]
Ant	85	25	70.30	<.0001[b]
Ac	40	45	.29	.5902

[a] Rf random flight, Of oriented flight, La landing, Ant antennating, Ac attempt copulation.

[b] Percents in the same row are significantly different according to a x^2 2x2 test of independence.

hairpencils) that are directed, after landing, to the female, and it is assumed that the hairpencils release a secretion which serves as an arrestant agent during courtship (Teal et al. 1981).

Acquiescence of the female, accurate parallel movement of the male, and the male's ability to clasp the female genitalia have been identified as requisites for successful mating in H. virescens (Teal 1981). In Spodoptera littoralis (Boisd.) the male's brush display, female wing lift (acceptance), female wing flick (rejection), and settling of the male beside the female were essential patterns to mating (Ellis & Brimacombe 1980). Leppla et al. (1987) described a bilateral antennation in A. gemmatalis, but the point of mutual recognition and acceptance was when the male made a full display of brushes and hairpencils. Unlike G. molesta, a tortricid, where males attracted females after they themselves have been attracted to the vicinity of a female (Baker et al. 1981), H. subflexa and H. virescens (Teal 1981) females did not take a more active part in the courtship.

A certain amount of flexibility in H. subflexa behavior was observed. Occasionally males paired successfully without antennating the female tip's ovipositor, making a rough landing, or forcing a copulation. However, results support the hypothesis that the males following the stereotypical behavior outlined in Figure 3-3 had a greater

probability of success in sexual reproduction. Overall, results indicated that, of the 43 tested, 27 mated successfully (62 percent). These results are in agreement with those of a similar study with H. virescens where 58 percent of the initial reproductive encounters resulted in successful matings (Teal et al. 1981). Ellis and Brimacombe (1980) found that 55 percent of the males of S. littoralis had courtships that led to copulation, and Leppla et al. (1987) reported that A. gemmatalis had 70 percent of successful courtships directly or after male cycling. Results from the present experiments as well as those mentioned above indicated that in these laboratory studies initial sexual encounters had a reasonable probability (0.5-0.6) that the final outcome would be successful.

Variation in individual mating success is documented. Such variation means that some individuals may never mate while others may mate many times (Partridge 1983). Halliday (1983) has defined mate choice as ..."any pattern of behavior, shown by members of one sex, that leads to their being more likely, to mate with certain members of the opposite sex than with others" (p. 4). In this context of sexual selection (Borgia 1979), identification of the male traits that females might use to discriminate among potential partners (such as antennating, thrust, display of brushes, etc.) would be relevant if it would be possible to explain how they contribute to female reproductive success.

There are other species in which males offer resources to females. For example, Drosophila suboscura Collins males provide a drop of regurgitated food during courtship and the females feeding on this material were observed to lay more eggs on an incomplete diet than females not receiving a drop (Steele 1986). However, with males that provide only an elaborate courtship like that described herein for H. subflexa it is more difficult to explain in terms of the female choice hypothesis, i.e., how a female can associate courtship features with male genetic quality. One response to this question would be that males transfer sperm in large numbers contained within associated fluids and/or spermatophores which may serve either to nourish the sperm or to provide stimulants or nutrients for egg production by the female (Davey 1985). For instance, male butterflies make a substantial paternal investment in their offspring in the form of nutrients (proteins, hydrocarbons, water) passed with the sperm into the female's reproductive tract during copulation. The protein component of the male's secretion was shown to be used by females for egg production (Boggs & Gilbert 1979), or protection of the offspring (Eisner & Meinwald 1986). Thus, females may discriminate among potential partners. Those males with the desirable traits will be selected, and in turn females are going to exert pressure to promote these characteristics in the following male generations.

For instance, in Utetheisa ornatrix (L.) it was unclear as to why females and males often engage in elaborate courtship behavior. The male is equipped with hairpencils that are thrust against the female's body during courtship. Eisner & Meinwald (1986) found that U. ornatryx hairpencils secrete a pheromone derived from a pyrrolizidine plant alkaloid. Because this substance is poisonous to many animals, pyrrolizidine protects the plants from being eaten. Males of U. ornatrix store this chemical in their genitalia and during copulation they pump out the compound in their seminal fluid, which is then incorporated into the female eggs, making the eggs unpalatable to predators. Hence, the male hairpencil display during courtship allows the U. ornatrix female to evaluate the fitness of her potential partner and "decide" (Wittenberger 1983) whether or not to mate. The same study showed that males reared on artificial diet without pyrrolizidines were rejected by females. Thus, male behaviors that occur in the mating sequence of H. subflexa may indicate some clues (antennating, proper parallel movement, adequate attempt copulation, male size?) to the calling female that presumably might benefit its efficiency in reproduction.

When comparing metabolic costs for production of sperm with production of eggs, it is clear (Dewsbury 1982) that the sperm's costs may be trivial relative to eggs. However, costs associated with the production of fluids and/or

spermatophores for sperm transfer may be expensive and limit the number of females a male can inseminate (Dewsbury 1982). Consequently, males may discriminate among females as potential mating partners. Therefore, mate-choice options are open to the female and male during the mating process. However, until there is more detailed information available on the extent to which the secretions imparted by H. subflexa males during copulation affect female reproductive capacity it will be difficult to assess the importance of the female or male choice hypothesis.

Identification of chemical constituents in the pheromone blend of an insect is part of the pheromonal research process. Description of the insect behavioral courtship sequence and identification of behaviors that are essential in the behavioral repertoire also are important. The present study of H. subflexa courtship behaviors provides behavioral parameters to make comparisons with backcross insects, and provides the background for understanding the relationship between chemicals and evoked behaviors. This association is discussed in Chapter 4.

Backcross insects. Mating experiments with backcross insects were characterized by a marked low activity within the male population. One indirect but very visible measure of activity observed was that males from parental species kept in holding cages for 4-5 days suffered a noticeable scale loss and/or tattering of one or both wings. Both

tattering and scale loss were viewed as a consequence of ambulatory and/or flight movements in the relatively reduced dimensions of the cages during the daily activity period. Males from backcross generations maintained their wings and associated scales in relatively good conditions because most of these insects did not engage in a similar high level of activity. Therefore, it was understandable why wings of the backcross males deteriorated less rapidly than those of the parental species.

The behavioral units identified in the courtship behavior of backcross males were: activation, Act; random flight, Rf; oriented flight, Of; landing, La; hairpencil display, Hd; move parallel, Mp; attempted copulation, Ac; mating, Ma; sitting, Sit; and female escape, Fe. Mated females were dissected removing the bursa copulatrix to verify the presence of the spermatophore.

The courtship behavior sequence began when a male was introduced into the wind tunnel which contained a virgin calling female. The males began to orient their antennae and vibrate their wings to initiate flight. Partial exposure of the hairpencils was a signal of pheromone perception. At this initial stage, some males ($P = 0.16$) remained in the release cage after brief activation. These males did not show any further response to the presumable flux of pheromone reaching their receptors. Previous bioassays with parental species indicated that, in most moth

populations, a few (2 to 5 percent) did not respond to the odor stimuli due to unknown causes (i.e., damage during handling, no appropriate physiological state, disease, etc.). These moths were discarded. Nevertheless, in preliminary behavioral observations using initial backcross breedings (BC_3 - BC_4), it was evident that a relatively high percent (20-25) of these males adopted the resting posture during bioassays. This behavior was assumed to be a characteristic of the male backcross population and was included in the behavioral sequence. Transitions between consecutive behaviors with $P < .05$ are depicted in Fig. 3-4. Some behavioral states were not included if they were either rare or deviated only slightly from chance occurrence.

Perception of female pheromone in the active space evoked random flight ($P = 0.47$) or oriented flight ($P = 0.37$). Random flight was characterized by turns and crosswind zigzagging. Males exhibited short positive phototactic reaction to the red lights that provided illumination in the wind tunnel. After a few seconds, most of the males in random flight flew upwind toward the pheromone source ($P = 0.67$). However, a relatively high proportion of these males were unable to detect the pheromone plume ($P = 0.30$) and rapidly settled on the wind tunnel walls. Orienting males flew in a zigzag path aligned along the plume with horizontal rather than vertical undulations. This characteristic pattern of insects flying

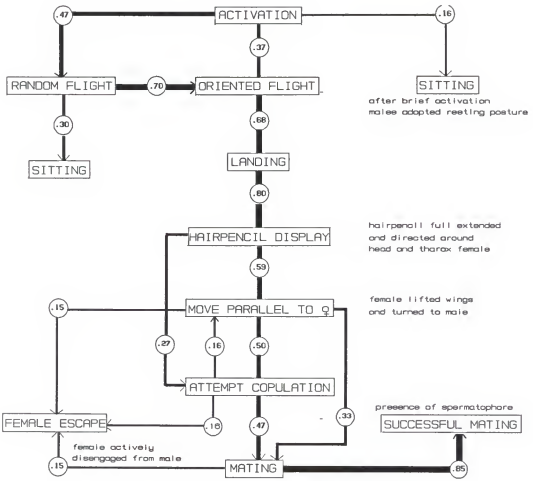

Figure 3-4. Flowchart of the courtship behavior sequence of backcross insects (BC_5-BC_6), ($P<0.05$). Thickness of lines and values in circles indicate the transition probability between two adjacent behaviors.

to pheromones has been attributed to reiterative losses of scents and turning back toward the direction in which the odor was last perceived (Shorey 1973).

Although the conditions in the wind tunnel did not duplicate the pheromone plume in the field--factors such as wind speed, vegetation, objects, etc., may give many form variations to the pheromone plume in the field--it offered an acceptable version of the probable moth movements under natural conditions.

Heliothis subflexa oriented males landed ($P = 0.68$) laterally or under the female with full exhibition ($P = 0.80$) of their hairpencils and directed them around the head and thorax of the female. These hairpencils were well developed, and the morphological insertion in the male genitalia corresponded to the characteristic profusion of hairs observed on the movable genital claspers and on the posterior end of the eighth segment of H. virescens males (Brazzel et al. 1953). The hairpencils of H. subflexa males are concentrated principally around the anterior end of the eighth abdominal segment. There are a few hairpencils on the claspers, but they are short and sparsely distributed.

Some of the males lost the plume during oriented flight and returned to random flight before settling on the wind tunnel wall; however, the transition (Of to Sit) did not differ from chance prediction ($P < 0.05$). At the time of hairpencil display, the male moved parallel to female's body

(P = 0.59), or he attempted copulation directly (P = 0.27). Heliothis virescens (Teal 1981) or backcross males did not antennate the female ovipositor as did the parental species H. subflexa. The hairpencil display showed by backcross and H. virescens (Teal 1981) likely contributed to female acquiescence and gave the male the opportunity to perform the next behavior in the sequence. Proper parallel alignment was necessary for copulation (P = 0.33) or attempted copulation (P =.50). Failure of males to adopt the position for clasping the female's ovipositor caused the female to leave (P = 0.15) by flying away or dropping from the screenwire. The male often was "trapped" by the scent mark left by the female on the surface of the sreenwire. Consequently, these unsuccessful males were unable to locate the female again during the recording period.

A portion of the males that attempted copulation from the appropriate position clasped the female genitalia and continued with mating (P = 0.47). Other males were actively rejected by females by curving the ovipositor upward and extensive wing fanning which frustrated male mating attempts. Rejecting females eventually stopped wing fanning and flew or walked away (P = 0.16) from the males. In pairs that reached the final stage of courtship, it was evident in some cases (P = 0.15) that females disengaged from their mates by extensive wing fanning, walking, and retraction of the ovipositor. Similar separation behavior was observed in

H. subflexa and H. virescens, but the reason for the break-off of copulation in both cases remains undetermined. During this separation the male's ability to track a moving female depended not only on his agility but also on the extent to which the female moved away from him.

Clearly, successful males that disengaged momentarily from the female were better at tracking females as they moved away, and they were able to resume copulation. Unsuccessful males appeared unable to keep up with the female and stopped pursuing her. Overall results indicated that of 51 initial pairs only 17 of them (33 percent) culminated the courtship sequence successfully. Thus, behavioral data presented here indicated that the backcross male's tendency to settle down after a limited flight activity contributed greatly to the increased proportion of unsuccessful males. This passivity was more evident in the initial stages of the sequence. After a brief activation, a total of nine (18 percent) males did not take flight, and a total of 11 (22 percent) males resettled on the wind tunnel walls after a brief random flight. Although this tendency to sit after activation or random flight was displayed by H. subflexa males, only a 3 to 5 percent of the sampled population was involved.

Other factors that contributed to the low level of the backcross reproductive success were failure to move parallel to the female and improper copulation attempt. These

behavioral determinants have been pointed out by Teal et al. (1981) as decisive behaviors in the sequence of H. virescens.

A comparison of the mean duration (sec) of the main behaviors observed in reproductive encounters of successful and unsuccessful backcross insects indicated that there were no significant differences among these groups (Table 3-3) in the duration of random and oriented flight, and landing arrival. However, unsuccessful insects spent more time either trying to copulate ($z = 3.4$, $P = 0.01$) or sitting ($z = 8.1$, $P = 0.001$) than successful ones.

Visual examination of the BC insects did not indicate any deformities or physical damage, and they were heavier ($P < 0.05$) in the pupal stage (338 ± 37.9 mg) than H. subflexa (229 ± 56.1 mg) or H. virescens (232 ± 49.2 mg) males. Probably, these differences were caused by rearing procedures. Backcross larvae were reared in individual 1 oz cups, and the parental species were reared in multicellular larval rearing units. Nevertheless, the performance of BC males in the wind tunnel was generally lower than comparable parameters exhibited by H. subflexa males.

In first generation hybrids, half the nuclear genes were from H. subflexa and half from H. virescens, but all self-replicating cytoplasmic elements (i.e., mitochondria) and the Z chromosome (males are ZZ) were from H. subflexa (LaChance 1984). Since H. virescens nuclear genes increased

Table 3-3. Comparison of mean duration (sec) of main behaviors observed in reproductive encounters of successful and unsuccessful backcross insects.

Behavior[a]	Successful n	Mean (S.E.)	Unsuccessful n	Mean (S.E.)	Comparison z	P
Rf	13	17.8 ± 2.4	11	26.8 ± 3.9	1.50	0.13
Of	17	11.4 ± 1.2	15	15.7 ± 1.9	1.47	0.14
La	17	36 ± 3.3	8	35.7 ± 3.3	.03	0.90
Ac	8	2.3 ± 0.3	11	5.0 ± 0.7	3.40	0.01 [b]
Sit	3	10.6 ± 4.2	24	64.1 ± 5.1	8.10	0.001[b]

[a] Rf random flight, Of oriented flight, La landing, Ac attempted copulation, Sit sitting.

[a] Means in the same row are significantly different according to the Mann-Whitney U-test.

by an expected 50 percent each generation of backcrossing, the BC_5 and BC_6 generations, at the time bioassays were made had 98.4 and 99.6 percent, respectively, of H. virescens nuclear genes, but had replicating cytoplasmic elements and a Z chromosome from H. subflexa.

In early backcross generations, meiotic disturbances in chromosome pairing have been reported that presumably cause sterility in the BC males (Goodpasture et al. 1980). Chromosome pairing at the first meiotic division is almost normal by the 6th backcross, as well as the disappearance of sperm with two tails. Therefore, the basis of sterility in males after the 6th backcross is due to causes other than meoitic disturbances (Goodpasture et al. 1980, LaChance 1984). Thus, it is possible to speculate that these abnormalities could influence negatively the overall physical performance of these early backcross insects. However, the fact that some males completed the courtship behavior successfully made this assumption weak, unless hybridization increased the probability of the appearance of genetic abnormalities that in normal conditions are restricted to a few insects. Abnormalities are often found in the degenerating sperm from the spermathecae of normal females mated to normal males (LaChance 1984).

Karpenko & Proshold (1977) suggested that persistence of sterility could lie within the female, since maternal effects could include both chromosomal and extrachromosomal

(cytoplasmic) factors. LaChance (1984) indicated that sperm abnormalities, such as swollen mitochondrial derivatives, degenerating bundles, and anomalies in the sperm tails contributed to sterility in the late backcrosses. A detailed biochemical approach by Miller et al. (1986) suggested that F_1F_o-ATPase complex in the sperm mitochondria of backcross males may be less efficient in producing the required energy (ATP) to complete development in these germinal cells and in turn, causing the BC male sterility. Consequently, nuclei-cytoplasm interactions in the backcross insect sperm predicted by Karpenko & Proshold (1977) help explain the persistence of sterility in backcross insects. Because the chromosomal background is not uniform through generations in these insects, evaluation of male behavioral performance in late generations may give another picture. Analysis of behavioral and chemical interactions between parental species and backcross insects as well as a discussion of associated published data are presented in Chapter 5.

CHAPTER 4

ROLE OF COMPONENTS OF HELIOTHIS SUBFLEXA PHEROMONE ON MALE COURTSHIP BEHAVIOR SEQUENCE

Introduction

Heliothis subflexa (Guenée), unlike some congeneric species, feeds exclusively on groundcherry fruits (Physalis spp.). In addition to the USA (Brazzel et al. 1953) and the Caribbean area (Garcia 1975), the insect occurs in Mexico where it is considered a pest of P. ixocarpa Brot., a cultivar with economic importance in that country (> 15000 ha in 1982, Chavez 1982).

The sex pheromone of H. subflexa has been identified (Teal et al. 1981a, Klun et al. 1982), and consists of a blend of aldehydes, acetate esters and alcohols. However, field experiments using sticky traps (Teal et al. 1981a) indicated that the cues responsible for close range behaviors (i.e., landing, attempt copulation, stay on stimuli source) were absent, masked or inhibited. This was evident in other evaluations when the synthetic blend was deployed in sticky traps; there was a significant decrease in males captured relative to captures in similar traps baited with virgin females (Mitchell and Heath, personal communication). Hence, an analysis of the relationship

between pheromonal chemicals and evoked behaviors in H. subflexa males would facilitate the identification of the compounds or mixtures that operate in those close-range behaviors.

The use of synthetic sex pheromones in insect pest management practices has been limited to a few species. Reasons for this are many and varied and they are discussed in detail by Mitchell (1986). A better understanding of the behavioral mechanisms by which components (individual or mixtures) of pheromonal blends cause sexual communication would enhance the use of pheromones in insect control strategies (Lewis 1981, Mitchell 1981, Palaniswamy & Underhill 1988, Cardé 1988, Minks & Cardé 1988).

The courtship behavior sequence of H. subflexa has been described in flight tunnel studies (Chapter 3). Therefore, in this Chapter I present the results of laboratory and field studies on the relationship between pheromone components and evoked behaviors in H. subflexa males.

Materials and Methods

Laboratory Studies

Insects. Heliothis subflexa was reared using methods described by Mitchell et al. (1988). Males were separated from females as pupae and kept under 25 ± 2°C, 14:10 light-dark photoperiod, and 50-70 % relative humidity. Adults were held in clear acrylic cages until tested.

Gland extracts. Ovipositors from calling females (2-4 days-old) were clipped under a dim red light (≈ 2 lux) and immersed in hexane for 45 seconds. For chemical analysis, individual glands were soaked in 25 µl hexane, and for bioassays, pooled samples were soaked in 100 µl per gland. The crude extracts were used without further concentration.

Chemistry. All synthetic material used was provided by the Chemistry Section of the IABBRL, USDA at Gainesville, FL. The compounds used were: tetradecanal (14:Al), (Z)-9-tetradecenal (Z9-14:Al), hexadecanal (16:Al), (Z)-7-hexadecenal (Z7-16:Al), (Z)-9-hexadecenal (Z9-16:Al), (Z)-11-hexadecenal (Z11-16:Al), (Z)-7-hexadecen-1-ol acetate (Z7-16:Ac), (Z)-9-hexadecen-1-ol acetate (Z9-16:Ac), (Z)-11-hexadecen-ol acetate (Z11-16:Ac), (Z)-9-hexadecen-1-ol (Z9-16:OH), and (Z)-11-hexadecen-1-ol (Z11-16:OH).

Synthetic pheromone blends used in field trials were formulated on rubber septa (#8153-022, A. H. Thomas Co. Philadelphia, PA). Each septum was loaded with 100 µl of a hexane solution such that the emission rates of the compounds approximated the emission rates from collected crude extracts. Stock solutions used in wind tunnel studies were made and stored at -20°C. Each filter paper was loaded with 100 µl of hexane containing chemicals in proportion to those found in the crude extract from one female (1 FE).

Release rate and the ratio of pheromone emitted was determined on septa aged in the laboratory as well as septa

used in the field experiments. The ratio and rate of release of pheromone from a septum was determined by placing the septum in a stainless-steel chamber; purified air (1 liter/min) then was passed over the septum. The volatilized pheromone was collected on a small filter prepared by sealing 50 mg of Super Q-80/100 mesh (Alltech Associates, Inc.) between two plugs of glass wool in a 4 cm long x 4.0 mm (ID) Pyrex tube. The Super-Q filter was placed at the exit end of the stainless steel chamber and one liter/min of filtered air was passed through the system. After one h (two h for septa with low concentration) of collection the filter was removed and rinsed with four aliquots (100 μl) of methylene chloride. Then 50 ng of C_{18} hydrocarbon (iso-octadecane) was added as an internal standard and the volume concentrated to 5-10 μl.

Quality, quantity and relative proportions of chemical in crude extracts, synthetic blends and collected materials were assessed with a Varian Series 3700 gas chromatograph equipped with a splitless column capillary injector and flame ionization detector. Synthetic compounds were evaluated individually and compared with the internal standard to verify pureness and concentration. Data from the Varian 3700 were acquired through a Nelson Analytical (760 series interface) data acquisition system and processed using an Hewlett Packard 9121 computer equipped with chromatography software. Columns used in GC analysis were a

fused silica capillary column 30 m x 0.25 mm ID SPB1 column (Supelco), and a 30 m x 0.25 mm ID Supelcowax (bonded Carbowax) column. The operating conditions were splitless purge at 0.5 minutes, initial temperature of 60°C maintained for two minutes and then increased at 10°C/min to a final temperature of 200°C. Helium was used as the carrier gas at linear flow velocity of 20 cm/sec. The retention times of the various components in the ovipositor extracts were compared with those of standard compounds on the two capillary columns.

Flight tunnel bioassays. The flight tunnel used in these tests was the same as described in Chapter 3. All of the tests were conducted with males (2-4 days-old) between hours 4 and 6 of the scotophase. The behaviors used to categorize the response of males to crude extracts were defined by monitoring male response to calling virgin females held in a screen cage placed at the center of the upwind end of the wind tunnel.

For evaluation of crude extracts or synthetic chemicals, a single filter paper circle (4.5 cm diameter), impregnated with 1 FE of material, was positioned in the upwind end of the tunnel and individual males were exposed to each treatment for two minutes. The filter paper, treated either with crude extract or synthetic pheromonal blend, was used to test the response of four insects, after which it was replaced with a freshly-treated filter paper.

Specific behaviors and their total duration (seconds) were recorded on a portable Epson® PX-8 computer using a time event-recorder program. Total mean time duration of behaviors were analyzed by analysis of variance. Least-squares means were compared using Duncan's multiple range test. Percentages of insects participating in specific behaviors were analyzed by using a x^2 2 x 2 test of independence.

Field Experiments

Field trials were conducted in Alachua, FL, during fall of 1987. At that time, there was a sufficient supply of wild groundcherry plants and H. subflexa population. Tests were carried out with sticky (Zoecon Corp.) and bucket traps (International Pheromone Moth Traps, International Pheromone Systems, Marseyside, England) using rubber septa as dispensers for the synthetic blends. To avoid cross-contamination, the septa were prepared in the laboratory and transported to the field in sealed glass vials where the traps were baited. Transfer of lures from vials to the traps was facilitated by color-coded pins inserted in the septa. The pins also helped avoid handling the pheromone dispensers directly.

Sticky traps baited with 2 females (replaced every two days) were used as controls. A 3 x 4 cm piece of No-pest® insecticide strip (Texize, Greenville, SC) was placed in the bottom of the bucket traps to kill captured moths.

Experiments with sticky traps were carried out in a completely randomized design with six treatments and 10 repetitions in test 1, and five treatments with three repetitions in test 2. Traps were positioned 20 m apart and randomized daily after insect collection. A basic mixture consisting of 16:Al, Z9-16:Al, Z11-16:Al, Z9-16:Ac and Z11-16:Ac (plus Z7-16:Ac in test 2) was loaded on septa. Additional chemical(s) were added on the septa to complete 0.5 mg in test 1, and 1 mg in test 2.

In experiment 3, increased amounts of Z11-16:OH (emitted percent = 0, 0.9, 1.6, 3.5, and 4.5) plus the six-component mixture were loaded on septa placed in sticky and bucket traps. Traps with females and blank traps were used as controls. Finally, the ratio of Z11-16:OH giving the best capture in the experiment 3 was used in experiment 4 for determining the appropriate load dose of synthetic blend for bucket traps. Treatment percent means for daily trap catch data were compared using Duncan's new multiple-range test at $P < 0.05$ for treatment differences. Regression analysis was used to assess the effect of different dosages of the test blend on male moth captures.

Results and Discussion

Chemistry

Chemical analysis of female extract in hexane showed seven peaks (Fig. 4-1). When compared with pure synthetic compounds in both columns, they were identified as

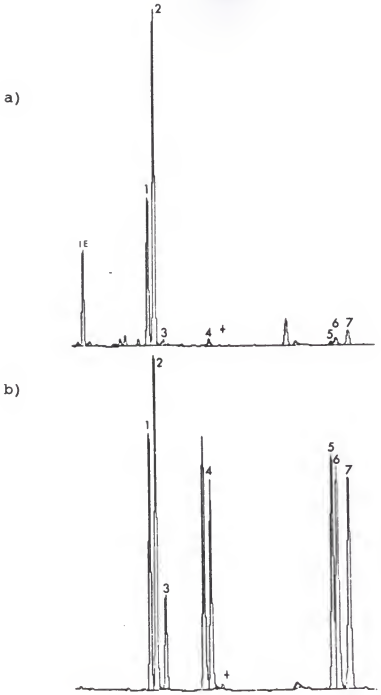

Fig. 4-1. Chromatograms of H. subflexa extract (1 FE) (a), and standard compounds (b) eluting from the SPB1 column. 1= (Z)-9-hexadecenal, 2= (Z)-11-hexadecenal, 3= hexadecanal, 4= (Z)-11-hexadecen-1-ol, 5= (Z)-7-hexadecen-1-ol acetate, 6= (Z)-9-hexadecen-1-ol acetate, 7= (Z)-11-hexadecen-1-ol acetate, IE= internal standard.

hexadecanal, (Z)-9-hexadecenal, (Z)-11-hexadecenal, (Z)-7-hexadecen-1-ol acetate, (Z)-9-hexadecen-1-ol acetate, (Z) 11-hexadecen-1-ol acetate, and (Z)-11-hexadecen-1-ol. Presence of (Z)-9-hexadecen-1-ol was no constant in the samples analyzed. This chemical composition is in agreement with the data of Teal et al. (1981a). In addition to the above compounds, Klun et al. (1982) found traces of 14:Al and Z9-14:Al.

Analyses of 15 individual female gland tips indicated that the mean percent composition of female sex pheromone was 16:Al, 0.98; Z9-16:Al, 25.02; Z11-16:Al, 60.14; Z7-16:Ac, 0.32; Z9-16:Ac, 2.52; Z11-16:Ac, 5.71; Z9-16:OH, .52; Z11-16:OH, 4.08; (Table 4-1). The total extracted pheromone from female ovipositor tips was variable (201.41 ± 12.48 ng/female). These percents and amounts of pheromone extracts were used to calculate the load ratio on septa required to provide the desired release rate of chemicals in the synthetic blend (Heath et al. 1986). Amounts and percents of chemical components of H. subflexa pheromone in crude extracts indicated by Teal et al. (1981a) differed from those reported here. Probable reasons for this difference were discussed in a recent paper by Heath et al. (1988). They observed that pheromone release by H. subflexa females was variable through the calling period, with the maximum liberation of chemicals in the last portion (≈ 40 min) of the calling period. Other factors such as

Table 4-1. Composition of hexane extracts from H. subflexa tip ovipositors.

Compound	Mean[a] (±S.E.) Composition (%)	Mean Weight/Female (ng)
16:Al	0.98 ± 0.10	1.86 ± 0.19
Z9-16:Al	25.02 ± 1.80	46.46 ± 4.75
Z11-16:Al	60.14 ± 2.04	122.63 ± 7.30
Z7-16:Ac	0.32 ± 0.03	0.59 ± 0.01
Z9-16:Ac	2.51 ± 0.37	5.19 ± 0.62
Z11-16:Ac	5.79 ± 0.77	14.54 ± 2.01
Z9-16:OH	0.49 ± 0.70	1.02 ± 0.07
Z11-16:OH	4.04 ± 0.46	6.88 ± 1.34

[a] n = 15

Table 4-2. Average release rate of H. subflexa female pheromone compounds from septa dispensers.

Compound	Original load	Initial emission	Release rate by septa (ng/h)	
	%	%	Aged 3 days[a]	Aged 12 days[b]
16:Al	1.3	1.1 ± 0.7	2.2 ± 4.1	2.4 ± 0.3
Z9-16:Al	22.9	28.9 ± 1.4	56.6 ± 4.0	50.8 ± 22.5
Z11-16:Al	53.4	61.5 ± 3.0	120.3 ± 10.7	111.1 ± 11.8
Z7-16:Ac	1.9	0.7 ± 0.4	1.3 ± 0.3	1.3 ± 0.4
Z9-16:Ac	5.3	2.0 ± 0.9	4.0 ± 2.7	4.3 ± 1.6
Z11-16:Ac	12.1	4.2 ± 1.9	8.2 ± 4.0	8.8 ± 3.3
Z11-16:OH	3.1	1.6 ± 0.7	3.1 ± 0.8	2.6 ± 0.8
Total amount emitted			195.7 ± 14.3	181.4 ± 16.8

[a] Kept in a fume hood.
[b] Aged under field conditions.

physiological state (Blum 1985), or female size (Mitchell, E. R., personal communication) may contribute to the variation in the pheromone production.

Comparison between septa aged 3 days in the laboratory and septa aged in the field for 12 days (Table 4-2), indicated that the release rate (ng/h) decreased by 8 percent (during 9 days). Thus, the total amount released by the septa varied from an initial 195.7 (± 12.2) to a final 181.4 (± 16.1) ng/h. However, the proportion of components in the pheromonal blend remained constant through the field exposure period (Table 4-3). Therefore, a reasonable release rate and ratio of components of H. subflexa female pheromone was obtained.

Table 4-3. Release ratio of H. subflexa female pheromone components from septa used in field experiments.

Compound	Percent Mean[a] (S.E.)	Range
16:Al	1.4 ± 0.4	1.0 - 2.6
Z9-16:Al	29.0 ± 1.2	26.6 - 31.1
Z11-16:Al	60.8 ± 1.9	56.4 - 62.5
Z7-16:Ac	0.7 ± 0.3	0.3 - 1.4
Z9-16:Ac	2.0 ± 0.5	1.1 - 2.9
Z11-16:Ac	4.4 ± 1.5	2.4 - 9.0
Z11-16:OH	1.5 ± 0.6	0.6 - 4.5

[a] Septa load = 0.3, 1, 3, 6, and 10 mg. Septa analysis was made after 3, 7, and 10 days in the field (n = 60).

Flight Tunnel Studies

Preliminary male behavioral observations showed that the filter paper discs loaded with 1 FE emitted the pheromonal compounds at a fairly constant rate for only 10 min. Thus, each dispenser loaded with a fresh 1 FE was used for only four males. Efforts to load crude extracts on septa were futile, because the normal pheromone amounts present in the crude extract were clearly insufficient to provide an adequate release rate. The responses evoked in males by the crude extracts loaded on filter paper were compared with those evoked by caged calling females, and the results are shown in Table 4-4.

Behaviors identified in these bioassays were random flight (Rf), oriented flight (Of), landing (La), and contact with the source (Co). Landing time was defined as the total time that the male walked or ran around on the chemical source (filter paper or cage), and it was used as a reference to evaluate pheromone constituents mixtures. Elapsed time from activation until landing on the source was considered as contact (Co).

Data analysis indicated that H. subflexa male behavior durations elicited by crude extract or the full synthetic blend were, in most cases, similar to those observed using calling virgin females in cages (Table 4-4). In these bioassays, the males did not attempt copulation, but clasper extension and hairpencil display by males upon arrival at

Table 4-4. Comparison of mean (S.E) total duration (sec) of behaviors of H. subflexa males in wind tunnel bioassays for crude gland extracts and the synthetic blend.

	Bait (1 FE)[a]		
Behavior	Female[b]	Crude Extract	Synthetic Blend
RF	13.1 ± 1.8 a	12.8 ± 1.9 a	9.3 ± 1.4 a
OF	14.8 ± 3.0 a	21.5 ± 2.3 b	21.9 ± 2.3 b
LA	75.8 ± 4.2 a	67.2 ± 3.4 a	72.9 ± 3.3 a
CO	15.5 ± 1.6 a	16.5 ± 1.1 a	17.3 ± 1.7 a

[a] Means followed by the same letter (rows) are not significantly different, Duncan's multiple-range test, $P < 0.05$ ($n = 20$).

[b] One female inside of a wirecage.

the stimuli source were observed. The role of 14:Al and Z9 14:Al in the regulation of H. subflexa sexual behavior, if any, could not be determined because behaviors evoked by synthetic blends without these compounds were similar to those evoked by crude extracts. These two aldehydes account for less than one percent in the total blend (Klun et al. 1982). Although the behavioral durations observed presumably have little meaning in the field, they did provide a means to differentiate the male's response to different blends, virgin females, or crude extracts.

Of primary importance is landing behavior; it is a prerequisite for mating under natural conditions and it has a decisive influence in catching males under (Tumlinson et al. 1982, Cardé, 1984). Identification of the specific cues that induce this behavior in H. subflexa may be useful in planning potential use of its pheromone.

For determining the role of individual pheromone components on male behavior the synthetic blend was produced eliminating one of the seven constituents from the total synthetic blend each time, and the resulting deficient blend was evaluated in the wind tunnel [subtraction protocol according to Cardé (1988)]. Results from these experiments were compared with those using filter paper baited with crude extract (Table 4-5).

Deletion of Z9-16:Al or Z11-16:Al from the chemical blend prevented the males from displaying any signal of

Table 4-5. Mean (± S. E.) total duration (sec) of behavioral responses of H. subflexa males to filter papers baited with different chemical compound mixtures (1 FE).

Behavior	Synthetic blend[a]	Chemical absent in pheromone blend[b]						
		Z9-16:Al	Z11-16:Al	Z7-16:Ac	Z9-16:Ac	Z11-16:Ac	Z9-16:OH	Z11-16:OH
RF	9 ± 1 a	57 ± 6 c	83 ± 7 d	14 ± 1 a	30 ± 5 b	53 ± 6 c	13 ± 2 a	47 ± 4 c
OF	21 ± 2 a	2 ± .1 c	4 ± .4 c	20 ± 2 a	26 ± 4 b	31 ± 4 b	19 ± 2 a	30 ± 3 b
LA	73 ± 3 a	0	0	47 ± 4 b	23 ± 3 c	18 ± 3 c	57 ± 3 a	10 ± 2 d
CO	17 ± 2 a	0	o	19 ± 2 a	30 ± 3 b	20 ± 3 a	16 ± 2 a	28 ± 3 b

[a] 16:Al, Z9-16:Al, Z11-16:Al, Z7-16:Ac Z9-16:Al, Z11-16:Ac, Z9-16:OH, Z11-16:OH.
[b] 20 flights/treatment, means followed by the same letter (rows) are not significantly different, Duncan' multiple-range test, P <0.05.

excitation ($P < 0.05$). During these bioassays nearly all the male moths flew randomly and only a few of them engaged briefly in oriented flight (Table 4-5). It was apparent that these compounds were a necessity for mate location.

Vetter & Baker (1983) reported Z9-14:Al and Z11-16:Al as the major mediators of chemical communication in H. virescens. It was obvious in these studies that Z11-16:Al and Z9-14:Al were essential for upwind flight to occur. Hence, the evidence suggests that the function of Z9-16:Al in H. subflexa behavior may be comparable to the role of Z9-14:Al in the sexual signaling system of H. virescens. Subtraction of any of these chemicals from the blends decreases significantly the probability of successful chemical communication in both H. virescens and H. subflexa.

The treatment blend which lacked 16:Al (1.01% in crude extract) was not significantly different in behavior durations ($P < 0.05$) and percent of landing males ($x^2 = 0.29$, $P = 0.59$) from the full component blend. Omission of 16:Al from the H. virescens pheromonal blend caused a significant decrease in close-range (landing, hairpencil display) behavior of males (Vetter & Baker 1983). However, exclusion of 16:Al from H. phloxiphaga's pheromone blend did not result in any obvious modifications of the behavioral response (Raina et al. 1986).

Based on Tamaki (1985), Z11-16:Al has been identified as part of the female-produced sex pheromone in 11 species

of Lepidoptera. This compound is commonly utilized in chemical communication by five species in the subfamily Heliothidinae. Data from Kehat et al. (1980), Klun et al. (1982), Vetter & Baker (1983), Raina et al. 1986, Teal et al. (1986) and from this study suggest that (Z)-11-hexadecenal may operate in conjunction with Z9-14:Al, Z9-16:Al, 16:Al, and Z11-16:OH in inducing attraction in the early phases of the courtship sequence.

For example, H. virescens males were induced to fly upwind by septa loaded with only Z9-14:Al and Z11-16:Al (Vetter & Baker 1983), or by virgin calling H. subflexa females. In the last case, most of the males failed to find the female (Teal 1981). In H. armigera Z9-16:Al and Z11-16:Al evoked upwind flight, but this combination was not as effective as females (Kehat et al. 1980). Hence, filter paper loaded only with Z9-16:Al and Z11-16:Al (ratio 1:2.7) evoked oriented flight and landing (55 percent) on the source. However, H. subflexa males remained only an average of 8.3 seconds on the filter paper and most of the time they hovered close to the source.

In addition to the ratio of Z11-16:Al to Z9-16:Al in the pheromone of H. subflexa, chemical signal specificity has been attributed to the presence of the Z9-16:Ac or Z11-16:Ac (Teal 1981; Klun et al. 1982). Results of subtracting a single acetate from the blend are shown in Table 4-5. Deletion of Z11-16:Ac, the main acetate, caused

only 65 percent of the males to land on the filter paper, and stay on the source an average of 18 ± 3 sec. By comparison, 95 percent of the test males landed and remained on the dispenser an average of 73 ± 3 seconds when it was loaded with the full synthetic blend.

Although subtraction of Z7-16:Ac produced a slight decrease in the landing time (average 47 ± 5 sec), the total durations of other behaviors were similar to those in the control treatment. Exclusion of Z9-16:Ac caused a significant delay in time for arrival to the source (30 versus 17 seconds in control, $P < 0.05$), and only 75 percent of the males arrived at the source and remained on the dispenser an average of 23 (± 3) seconds. There were no significant differences among treatments in the extension of claspers and display of hairpencils. Usually males that flew to the dispenser displayed their genitalia, but they never attempted to copulate with the source.

Overall, experiments where an individual acetate was deleted resulted in a significant decrease in the percent of males landing on the dispenser and in the duration of time that they remained on the source ($P < 0.05$). Since some males were able to orient, land, stay on source, and display their genitalia, individual deletion of the acetates did not prevent totally the manifestation of behaviors under wind tunnel conditions.

Analysis of the results of subtracting Z9-16:OH from the blend indicated that this treatment was not significantly different ($P < 0.05$) from the control treatment (Table 4-5). Apparently, Z9-16:OH did not have an appreciable influence on the behaviors of H. subflexa males.

Percent of males landing on the source baited with synthetic blends without Z11-16:OH was significantly different from the percent of males landing on filter paper lure with the full chemical mixture ($x^2 = 40.26$, $P < 0.001$). The total time that the males remained on the source was significantly different ($P < 0.05$) from the time spent by males on dispensers loaded with the full synthetic mixture. Consequently, male moths spent more time in random and oriented flight and hovering close ($\approx$ 10 cm) to the source than moths stimulated by the complete pheromonal mixture (Table 4-5).

Individual males did not attempt copulation with filter papers baited with either crude extracts or synthetic blends. However, when two males were released simultaneously in the wind tunnel, upon arrival at the source they tried to copulate with each other. These observations suggest that other cues (visual or tactile) may contribute to close-range behavior.

Although removal of a single acetate or Z11-16:OH from the synthetic pheromonal blend did not interrupt completely the courtship behavior in males, it reduced significantly

the percent of males landing and the duration of the time they remained at the source. A simple summary of chemicals and associated behaviors would be: Z9-16:Al and Z11-16:Al (ratio 1:2.7) induced males to take flight and begin to search the stimuli source. At the same time or later in the sequence, odor stimuli generated by the presence of acetates plus Z11-16:OH maximized the signals in the chemical background for locating the source, induced landing, and stimulated subsequent search on the source. Extrusion of claspers and display of hairpencils initially triggered by the aldehydes was reinforced by acetates and Z11-16:OH odors. Elapsed time from contact with the source until the moth left the dispenser was influenced by the presence of the main two acetates (Z9-16:Ac and Z11-16:Ac) and Z11-16:OH. The attempted copulation behavior may be reinforced by visual and/or tactile cues.

Since wind tunnel conditions do not cover all factors implicated in a natural environment (Wolfson 1988), field experiments were established to test the mixtures. The working hypothesis for field experiments was that the addition of Z11-16:OH (considered previously as an inhibitory compound), in the percent found in the extracts, to the pheromonal blend (aldehydes plus acetates) would attract males in the same proportion as virgin females.

Field Studies

Results of field experiments to determine the attractiveness of different pheromonal blends to H. subflexa males are shown in Table 4-6. In experiment 1, all traps baited with synthetic material had a common basic mixture consisting of: 16:Al, Z9-16:Al, Z11-16:Al, Z9-16:Ac, Z11-16:Ac. Moth captures with this mixture plus Z11-16:OH were significantly greater ($P < 0.05$) than captures from traps baited with blends without this alcohol (Table 4-6). Addition of either Z7-16:Ac or Z9-16:OH to the basic mixture did not improve captures of males. However, wild H. subflexa males were attracted in significantly greater numbers ($P < 0.05$) to traps baited with two virgin females (Table 4-6).

In experiment 2, septa were loaded with a total of 1 mg of a six-component blend (16:Al, Z9-16:Al, Z11-16:Al, Z7-16:Ac, Z9-16:Ac, Z11-16:Al) formulated to provide a release ratio similar to that found in the female glands. Either Z9-16:OH or Z11-16:OH was added to the six-components blend. Data from these field trials indicated that the addition of Z9-16:OH did not cause a significant increase ($P < 0.05$) in trap catch.

Addition of Z11-16:OH to the synthetic mixture significantly ($P < 0.05$) increased male capture. Inclusion of this alcohol in baits yielded trap catches that were indistinguishable from captures in traps baited with virgin

Table 4-6. Captures of H. subflexa males in Pherocon 1C sticky traps baited with a basic blend[a] plus additional components. Alachua, FL. 1987.

Z7-16:Ac	Z9-16:OH	Z11-16:OH	14:Al Z9-14:Al 16:Ac	Mean males/ night	Mean%[b] males/ night
Experiment 1 (septa load = 500 µg)[c]					
--	--	--	--	1	2 a
**	--	--	--	1	1 a
--	--	**	--	14	21 b
**	--	**	--	16	24 b
**	**	**	--	13	18 b
2 females				24	34 c
Experiment 2 (septa load = 1 mg)[d]					
**	**	--	--	4	5 a
**	--	**	--	15	22 b
**	**	**	--	18	24 b
**	**	**	**	19	26 b
2 females				17	23 b

[a] 1.3% 16:Al, 24.3% Z9-16:Al, 54.3% Z11-16:Al, 4.2% Z9-16:Ac, 13.9% Z11-16:Ac. Presence of a specific compound in the synthetic blend is indicated by **.

[b] Mean percents followed by the same letter are not significantly different, Duncan's multiple-range test, $P < 0.05$.

[c] Captures from 10 nights.

[d] Captures from 4 nights.

females (Table 4-6). Therefore, the presence of the Z11-16:OH in the bait was a decisive factor in inducing male moths to land on the sticky traps, a necessary requisite for trapping them. Addition of 14:Al, Z9-14:Al, and 16:Ac (Klun et al. 1982) to blends containing the basic mixture plus Z11-16:OH did not improve captures of H. subflexa males (Table 4-6).

In experiment 3, a blend ratio consisting of the basic 5-components with increased amounts of Z11-16:OH (percent of total emitted = 0, .9, 1.6, 3.5, 4.5) loaded on baits of sticky and bucket traps, indicated that more males were captured in the traps baited with 0.9, 1.6, and 3.5% of this alcohol (Fig. 4-2). The mean percent of moths caught when 1.6% of the alcohol was emitted was significantly higher ($P < 0.05$) than the mean percent trapped when the septa released no alcohol or when the alcohol released was 4.5 percent of the total emitted blend. Thus, in addition to the 6 component blend a precise release ratio of Z11-16:OH (between 1 and 3.5 percent) was required to maximize trap capture.

The effect of release rate of the six-component mixture, with the addition of Z11-16:OH (1.6 percent emitted) from rubber septa on male capture was investigated using bucket traps. Septa containing a total amount of 0.3, 1, 3, 6, and 10 mg of pheromone were used and provided a release rate range of 105 to 1110 ng\h. The results (Fig.

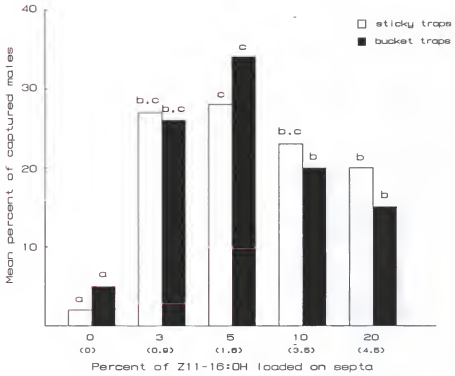

Fig. 4-2. Percent of male H. subflexa caught in sticky traps (open bars) and bucket traps (closed bars) baited with 6 pheromone components plus increased percents of Z11-16:OH. Values in parenthesis indicate percent of alcohol emitted. Bars with the same letter are not significantly different, Duncan's multiple-range test, $P < 0.05$.

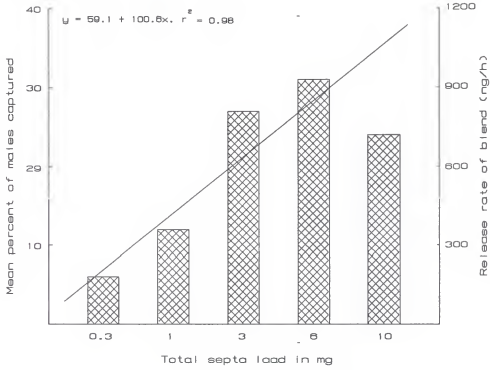

Fig. 4-3. Average number of male H. subflexa trapped per night in bucket traps baited with the 7 component pheromone blend at increased doses. Solid line is the release rate of pheromone (right axis).

4-3) showed a linear increase ($y = 8.35 + 4.23*X$, $r^2 = 0.88$) in trap captures as the septa load was increased to 6 mg of pheromone (590 ng/h released). The 10 mg load resulted in a reduction of males captured.

The Z11-16:OH is a common component in the pheromone gland extracts of almost all Heliothis species for which the sex pheromone has been identified; however, its role in the behavioral repertoire of these species has not been completely elucidated. Studies in H. virescens by Sparks et al. (1979) and Hartstack et al. (1980) indicated that the addition of Z11-16:OH to other components in the blend (cotton wicks used as dispensers) improved captures of males under field conditions. Nevertheless, when dispensed in rubber septa or hollow fibers, the addition of this alcohol did not increase trap catch (Hartstack et al. 1980). Laboratory experiments with H. virescens indicated that Z11-16:OH may not contribute to sexual communication in this species (Pope et al. 1982, Vetter & Baker 1983, Teal et al. 1986).

Field experiments by Raina et al. (1986) showed that traps baited with Z9-16:Al (0.5%), Z11-16:Al (91.8%) plus Z11-16:OH (2.9%) significantly enhanced trap catch of H. phloxiphaga males. Exclusion of this alcohol from the synthetic mixture reduced significantly male captures in the field thereby confirming early results from wind tunnel studies. Therefore, in H. phloxiphaga Z11-16:OH is a major

component mediator for chemical communication. Conversely, addition of Z11-16:OH (> 1%) to the synthetic blend of H. zea caused a significant reduction in the number of males captured when compared with trap catch in wire-cone traps baited only with Z9-16:Al and Z11-16:Al (ratio 4:96) (Teal et al. 1984).

In field trapping tests, Teal (1981) noted that when the complete blend of H. subflexa was loaded in polyethylene vials, total catch on sticky traps was reduced significantly. Further, considerably more males were captured in sticky traps when the alcohols were deleted, suggesting that the alcohols may act as a landing inhibitor. However, in those tests the mean percent compositions of Z9-16:OH and Z11-16:OH were identified as 14.4 and 12.2 %, respectively. Clearly, these alcohols were dispensed at ratios far above gland extracts. Tumlinson et al. (1982) predicted that the alcohols would not be inhibitory to landing if their release ratios were similar to those found within the gland. Data from this study confirmed that prediction and is in agreement with Klun et al. (1982) as well.

Although the release ratio of components loaded on septa was calculated from gland extracts, captures of H. subflexa males by traps baited with synthetic lure matched captures by caged female-bait traps. Therefore, it was assumed that the identification of insect pheromone was

complete. Because the release of synthetic material occurred all the time, it was possible that trap catch in traps baited with synthetics was influenced by a broad activity rhythm of males as discussed in Chapter 3. Visual observations at the time of captures (Cardé & Elkington 1984) or synthetic-baited traps equipped with an automatic sample changing device (Tingle et al. 1978) may help discern the efficacy of synthetic material in comparison with females.

For trapping studies, a precise release ratio and rate of the components of a sex pheromone was required to obtain male (or female) captures similar to those evoked by the opposite sex. To date formulations effective in achieving this goal have been very limited (Baker et al. 1980, Buttler & McDonough 1981, Heath et al. 1986, Cardé 1988). Ideally, the range of pheromone emission will be between a natural emitter and the upper rate effective in luring a male (Cardé 1988). Because of the difficulty involved in measuring the ratios, this range is rarely determined. However, it is recommended that different blends and release rates be screened in a volatile collection system before synthetic lures are used under field conditions.

Understanding the functional role of individual components (or combined) and their effect(s) on the behavioral repertoire of the target sex would be helpful in deciphering the precise mechanisms involved in the sexual

chemical communication within species. This basic chemical-behavior knowledge eventually would aid the development of pheromones in pest management strategies.

CHAPTER 5

BEHAVIORAL AND CHEMICAL INTERACTION BETWEEN BACKCROSS INSECTS AND PARENTAL SPECIES

Introduction

Crossing of Heliothis subflexa (Guenée) females and H. virescens (F.) males produces sterile hybrid males. Recurrent backcrossing of hybrid fertile females to H. virescens males perpetuates a sterility factor in the male progeny (Laster 1972). Release of these backcross insects has been proposed as a technique for managing populations of the tobacco budworm (TBW) (Makela & Huettel 1979).

The sexual performance of the hybrid progeny has been the subject of considerable investigation. Backcross (BC) female competitiveness for mating with H. virescens or BC males has been documented in laboratory (Karpenko & Proshold 1977, Pair et al. 1977), and field studies (Carpenter et al. 1979, Laster et al. 1978, Tingle et al. 1978, Proshold 1983, Martin et al. 1984). Performance of the BC males for mating with either conspecific or H. virescens has been variable. Pair et al. (1977) reported that matings with BC males apparently did not stop calling behavior of BC or TBW females because they mated more often than females mated with H. virescens. Karpenko & Proshold (1977) showed that

parental females crossed with BC males received a greater number of spermatophores than females crossed with conspecific males. Raulston et al. (1979) found that native males mated normally with backcross females, but BC males were observed only rarely mating with native females.

An extensive study by Proshold et al. (1983) in St. Croix, U.S. Virgin Islands indicated that released BC males were able to compete with H. virescens males for mates with virgin clip-winged females. Although the BC progeny survived the dry season, their numbers declined after BC releases regardless of the season. Proshold (1983) hypothesized that some type of selection might be operating against these backcross insects.

Although the cited reports on the potential use of hybrid sterility as a control strategy for the tobacco budworm (TBW) are encouraging, further studies are needed to evaluate the sexual competitiveness of the hybrid progeny. The objective of this study was to document the behavioral and chemical interactions between backcross Heliothis insects and the parental species in the laboratory under simulated flight conditions in a wind tunnel experiments.

Materials and Methods

Insects. A laboratory culture of H. subflexa was established as described by Mitchell et al. (1988). H. virescens pupae were received from Oxford, NC, on a weekly basis. They were sexed and held in separate environmental

chambers. Hybrids and backcrosses were produced from these cultures using the procedures described in Chapter 3.

Chemical analysis. Abdominal tips (eighth and ninth segments) of actively calling H. virescens, H. subflexa, hybrid, and backcross females (F_1, BC_3, BC_6, BC_9,) were clipped and immersed in hexane for 45 seconds. The washes were fairly clean extracts, so they could be analyzed by gas chromatography or used for wind tunnel bioassays.

Analyses were performed using a Varian model 3700 GC equipped with a splitless injector system and a flame ionization detector. A Carbowax 30 m x .25 mm (ID) glass capillary column was used. The operating conditions were splitless purge at 0.5 min, initial temperature of 60°C maintained for two minutes and then increased at 10°C/min to a final temperature of 200°C. Helium was used as the carrier gas at linear flow velocity of 20 cm/sec. The retention times of various components in the ovipositor extracts were compared with authentic samples. With the carbowax column, elution of Z7-16:Al and Z9-16:Al was coincident.

Wind tunnel studies. For evaluating extracts, a single filter paper (4.5 cm diameter) was positioned in the upwind end of the wind tunnel, and individual male moths were flown to each treatment. A new filter paper with crude extract was used every four insects. Moths 48-120 h postemergence from the parental species and from BC_4, BC_8 and BC_{12}

generations were used. Male sets (20 moths per treatment) from these groups were flown to female extracts of their respective species as well as to extracts from the others. Although main events of the courtship behavior were recorded as described previously (Chapter 3), landing time was used to evaluate male performance to crude extracts.

To assess the ability of virgin females (parental species and BC_{13}) to attract and to mate with intra-interspecific males, groups of five females were introduced at the upwind end of the flight tunnel. After the females settled down, individual males were released at the downwind end of the tunnel. Successful matings were recorded and analyzed by a x^2 2 x 2 test of independence.

Initial efforts to set up a dual-selection bioassay using two virgin females (H. virescens and BC) were hampered because often one of the females stopped calling and the released male was unable to detect that female. Thus, it was unclear if the male's choice for landing was on the basis of the released pheromone or because only one of the females was calling. Therefore, groups of ten females (either H. virescens or backcross) were released in the wind tunnel. After females settled ten H. virescens and ten BC_{14} males were released simultaneously into the wind tunnel. The backcross males' dorsal prothorax (scales flaked off) were marked with Gloss Enamel (Walker and Wineriter 1981). After one hour, coupled moths were identified and kept in

individual vials for the remaining scotophase. The next day, females were dissected to determine the presence of a spermatophore in the bursa copulatrix. Results were analyzed by a x^2 2 x 2 test of independence ($P < 0.05$).

To assess the BC fertility, BC_{15} progeny were crossed or backcrossed with conspecific or parental species. Crosses were set up as follow: H. subflexa ♀ x H. subflexa ♂, H. virescens ♀ x H. virescens ♂, BC_{15} ♀ x BC_{15} ♂, H. subflexa ♀ x BC_{15} ♂, H. virescens ♀ x BC_{15} ♂, BC_{15} ♀ x H. virescens ♂, BC_{15} ♀ x H. subflexa ♂. Groups of 10 newly emerged pairs were confined for 5 days in gallon cartons as described by Mitchell et al. (1988). At the end of the experiment, females were dissected and spermatophores counted. One hundred eggs were selected randomly from each group, and the percent hatchability evaluated.

Results and Discussion

Analyses of Backcross Ovipositor Extracts

Analyses of hexane washes of backcross single ovipositors showed that the material from these females had a comparable chromatographic profile to that detected in washes from H. virescens females (Fig. 5-1). Although quantitative variations (Table 5-1) among females were observed, six chromatographic components were consistently detected ((Z)-7-hexadecenal/(Z)-9-hexadecenal, no resolution). The retention times of components were coincident with the retention times of authentic samples of

(Z)-9-tetradecenal, tetradecanal, hexadecanal, (Z)-7/9-hexadecenal, (Z)-11-hexadecenal, and (Z)-11-hexadecen-1-ol.

In comparison with H. virescens or backcross females, there was a significant difference in the production of (Z)-9-hexadecenal by hybrid females ($F = 14.32$, $P < 0.001$). The ratio of this compound with (Z)-11-hexadecenal constitutes a clear difference between parental species (Klun et al. 1982, Teal et al. 1986). In the present study, H. subflexa had a Z9-16:Al:Z11-16:Al ratio of 1:2.6; in contrast, H. virescens' ratio was approximately 1:43. Hybrid female washes gave a ratio of 1:5.6. Backcross female washes gave ratios of these two aldehydes comparable (1:38) to those from H. virescens extracts. There were no significant differences in the composition of the other chemicals in the pheromone glands (Table 5-1). Thus, the backcross females produced, at least through 9 generations, pheromone components similar in quality and quantity to the six-component mixture detected in H. virescens ovipositor washes. These results are in agreement with data for F1 and BC_{34} females reported by Klun et al. (1982).

Wind Tunnel Bioassays

Backcross male response to crude extracts

Hybrid (F_1) males and the first three generations of backcross males were heavier (>320 mg) than males from later generations (Fig. 5-2). Pupal weight of late (> BC_6) backcross males (avg = 276 ± 14 mg) was in the range of H.

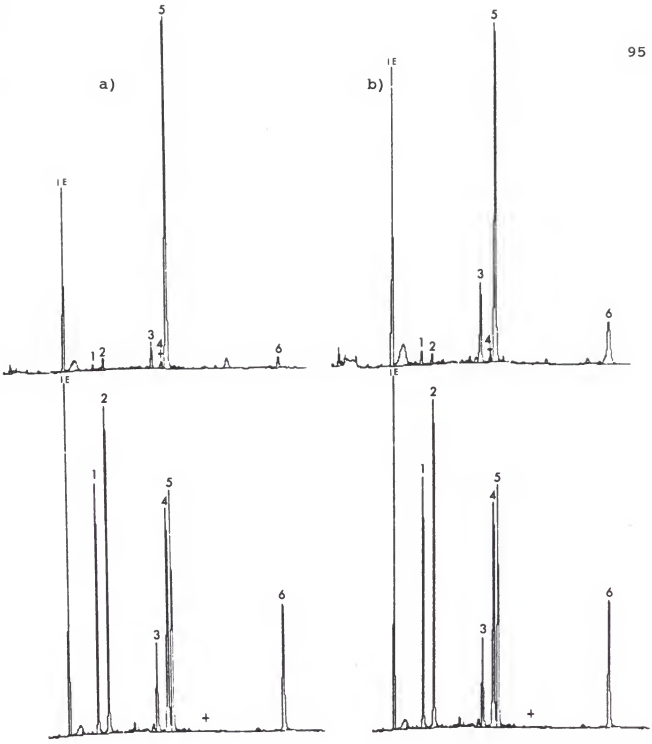

Fig. 5-1. Chromatographic profile of H. virescens (a) and backcross (b) tip ovipositor washes. Standard test mixture is showed below each sample (Carbowax column). 1= tetradecanal, 2= (Z)-9-tetradecenal, 3= hexadecanal, 4= (Z)-9-hexadecenal, 5= (Z)-11-hexadecenal, 6= (Z)-11-hexadecen-1-ol. IE= internal standard.

Table 5-1. Percentage composition of lipid components in hexane washes from H. virescens and selected backcross female ovipositors determined by GC analysis.

Chemical[a]	Mean Percent Composition (± S.E.)				
	H. virescens (18)[b]	F1 (11)	BC-3 (12)	BC-6 (12)	BC-9 (12)
14:Al	2.2 ± .2	2.4 ± .3	2.2 ± .4	2.1 ± .3	3.1 ± .2
Z9-14:Al	1.9 ± .2	2.8 ± .5	3.1 ± .4	1.7 ± .3	3.5 ± .4
16:Al	9.4 ± .9	7.2 ± .8	11.1 ± .8	8.1 ± .7	8.7 ± .9
Z9-16:Al	0.9 ± .2	7.6 ± .9	1.1 ± .3	2.7 ± .9	2.9 ± .5
Z11-16:Al	82.1 ± 1	74.5 ± 1	74.4 ± 2	79.2 ± 1	76.2 ± 2
Z11-16:OH	3.3 ± .5	4.5 ± .5	2.8 ± .9	5.7 ± .8	4.5 ± .4

[a] 14:Al= tetradecanal, Z9-14:Al= (Z)-9-tetradecenal, 16:Al= hexadecanal, Z9-16:Al= (Z)-9-hexadecenal, Z11-16:Al= (Z)-11-hexadecenal, Z11-16:OH= (Z)-11-hexadecen-1-ol. Internal standard, C_{18} hydrocarbon.

[b] number of ovipositors analyzed shown in ().

virescens male weights reared individually in 1 oz cups (avg = 287 ± 23 mg). An increase in weight and size usually occurs when two different species can break through initial reproductive barriers. This "hybrid vigor" has been related to the segregation of deleterious recessives that were homozygous in the original species, and heterozygote advantage at many loci in the hybrids (Campbell 1987). Despite their apparent strength, the initial backcross males (BC_6) displayed limited intervals of activity during the scotophase. Males from the later generations exhibited flight periods comparable to those exhibited by parental species.

Backcross male behaviors evoked by crude extracts loaded on filter paper included oriented flight, landing on source, hairpencil display, and attempted copulation. Like H. virescens males (Teal 1981), while backcross males curved their abdomens in an attempt to copulate with the stimuli source. Of these behaviors, landing time (defined as total time that the moth spends on the source) was selected to establish the association, if any, between crude extracts and an evoked landing response in males. Usually when the moths arrived at the source they had already perceived the pheromone and oriented to the stimulus. Although the landing time behavior was not an adequate measure of the male mating ability, it provided an initial index for analysis of the response.

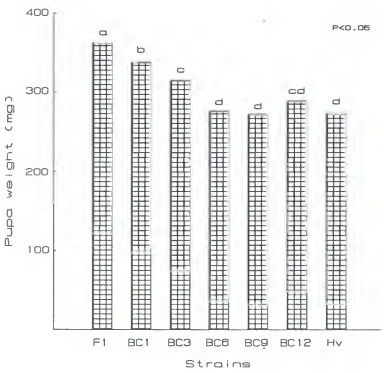

Figure 5-2. Pupal weight of H. virescens and selected backcrosses males reared in individual cups (n = 60). Bars with the same letter are not significantly different, Duncan's multiple-range test, $P < 0.05$. F_1 = hybrid, BC = backcross, Hv = H. virescens.

Data in Table 5-2 show the mean total time spent by parental species and selected backcross males on the filter paper. There were no significant differences ($F= 2.046$, $P > 0.1$) among the landing times of H. virescens males attracted by conspecific and backcross females extracts. In addition, the percent of H. virescens males landing was fairly constant, and no significant differences between treatments ($x^2 = 6.55$, $P > 0.05$) were observed. Landing response in the tobacco budworm male was not elicited by H. subflexa female crude extract. Approximately 25% of H. virescens detected and remained in the pheromone plume for a few seconds, but they were unable to locate the source. Common pheromone components (i.e., Z9-16:Al, Z11-16:Al) may induce oriented flight toward the source in both species, but males are still optimally responsive to the natural blend when given a choice (Teal 1981).

As expected, H. subflexa males responded positively (mean landing time = 73.3 sec) to crude extracts from conspecific females. About 20 percent of the H. subflexa males responded to filter paper baited with pheromone extract from BC_4 females. They remained on source for an average of 8.3 seconds; however, it was not possible to distinguish if this response was elicited by a low male threshold to common pheromone components (i.e., detection of Z9-16:Al/Z11-16:Al) or caused by the appearance of H. subflexa components in the crude extract loaded on filter

Table 5-2. Total mean time spent (sec) by H. virescens, H. subflexa, and selected backcross males on filter paper baited with different extracts.

	Mean total time spent by males[a]				
Extract[b]	Hv[c]	Hs[d]	BC-4[d]	BC-8[d]	BC-12[d]
Hv	36.1 a	0	16.6 a	37.2 a	39.7 a
Hs	0	69.4 a	0	0	0
BC-4	24.4 a	7.7 b	21.2 a	-	-
BC-8	38.7 a	0	-	26.4 a	-
BC-12	41.1 a	0	-	-	56.6 a

[a] n = 20 insects per treatment. Means (in columns) followed by the same letter are not significantly different ($P < 0.05$).
[b] Hv = H. virescens, Hs = H. subflexa, BC = backcross.
[c] Duncan's multiple range test.
[d] t-test.

paper. Chemical analysis of BC_3 (the previous generation) female extract did not reveal the presence of any acetates or a (Z)-9-hexadecenal/(Z)-11-hexadecenal ratio comparable to the ratio in H. subflexa that could evoke the landing response.

Fifty and sixty percent of the BC_4 males responded to crude extracts from TBW and conspecific females, respectively. The average landing times evoked by these two extracts were not significantly different (Table 5-2). Males from BC_8 (65 percent) and BC_{12} (70 percent) landed on filter paper baited with extracts from the conspecific backcross and H. virescens females. There were no significant differences in the landing time between these later backcross moths and H. virescens males (Table 5-2).

Comparison of BC_4, BC_8, and BC_{12} male responses evoked by TBW female extracts revealed significant differences ($F = 4.27$, $P < 0.01$). BC_4 males stayed on the source less time (16.6 ± 6.2 sec) than males from either the later backcrosses or from the TBW culture ($\approx$ 38 sec).

Results reported here, and those from the mating behavior studies (Chapter 3), suggest that initial backcross males may not be as competitive as later generation males (BC_6). The response by BC_{12} males to crude extracts from BC_{12} females supports the above statement. Landing time of these males (Table 5-2) was significantly different from all other treatments (when a backcross male was included). Possible

reasons for this behavioral response fluctuation between early and late generations are discussed below.

Goodpasture et al. (1980) reported that F_1 and early backcross males had meiotic and spermatogenic abnormalities These anomalies disappeared in the later backcross (> BC_6) generations. Thus, the possibility exists that the cause of these abnormalities may influence the overall performance of the early backcross generations. Continuous segregation of H. subflexa chromosomes and their replacement with H. virescens homologous (LaChance 1984) would contribute to make later backcross generations able to perform courtship behavior similar to males from parental species though the males remained sterile.

Another possibility is that unknown selection pressures in the laboratory (Leppla & King 1984, Wolfson 1988) might have influenced the performance of initial backcross breedings. As the colony passed through generations, and adapted to laboratory conditions, the resultant selected insects (i.e., BC_{12}) might be able to respond more actively to conspecific females than H. virescens females (Table 5-2). Female competitiveness from early backcrosses for attracting H. virescens males was comparable to that exhibited by TBW females.

Experiments in cotton fields by Raulston et al. (1979) indicated that released backcross males were non-competitive in mating with native females. These researchers suggested

that selected strains be used to "manufacture" BC cultures to suit the requirements (insect behavior, type of host, temperature, photoperiod, etc.) of the projected release area. Carpenter et al. (1979) reached a similar conclusion. They proposed that the mating behavior of BC insects may vary according to the host plant involved, and it may be possible to rear insects programmed for desirable behavioral traits.

Evidence from this study and from published data indicated that sexual attractiveness of BC females (early or later generations) was comparable to that observed in H. virescens females. Therefore, the selection pressure (if any) from laboratory conditions on BC insects was restricted to males.

BC male response to females: individual release.

There was no significant difference between the proportion of BC_{14} males mating with conspecific females and the percent of these males mated with TBW females (x^2 = 0.40, $P > 0.1$). However, when H. virescens males were tested with BC_{14} females, there was a significant difference in the percent of successful matings ($x^2 = 7.93$, $P < 0.05$) in comparison with crosses of BC_{14} x BC_{14} (Table 5-3). The mating of H. virescens males exposed to conspecific females was identical, statistically, to the proportion mating in the cross involving H. virescens males x BC_{14} females.

Table 5-3. Percent of successful matings of male moths with conspecific and parental species.

Male[a]	Female		
	BC_{14}	H. virescens	H. subflexa
BC-14	75 a	70 a	10 a
H. virescens	55 b	60 a	5 a

[a] Percentages in the same column with different letters are significantly different, x^2 2 x 2 test of independence ($P < 0.05$), 20 flights per treatment.

Females from H. subflexa elicited brief periods of oriented flight in H. virescens and BC_{14} males, but most of these males were unable to locate the position of the female. Approximately ten percent of the BC_{14} males were attracted to and mated with H. subflexa females. Cross attraction between these two closely related species has been observed under field (Tingle et al. 1978) and laboratory conditions (Teal et al. 1986). Given these considerations, the experimental, no-choice situation in a limited space (wind tunnel dimensions) most certainly increased the probability of these sexual encounters.

The significant difference in the percent of successful pairings between BC_{14} and TBW males mated with BC_{14} females suggests that the colony, through generations, enhanced its pheromonal communication. It is unknown if the increased behavioral response was caused by a BC population experiencing differing selection pressures (TBW was received every week as pupae) in the laboratory or caused by the continuous backcrossing with H. virescens. However, it was clear that later generations of BC insects, in a one-choice situation, performed better than their early counterparts.

BC males response to females: simultaneous release.

Enamel mark on the prothorax of the BC_{15} male resisted wear during the experiments, and apparently did not alter the behavior of the insect. Of 30 BC_{15} and 30 TBW females, 24 and 22, respectively, mated during the simultaneous male

Table 5-4. Percent of BC_{15} or TBW males, released simultaneously, that mated with conspecific and parental females under wind tunnel conditions.

	Percent of mated males[a]	
Female	BC_{15}	H. virescens
BC-15	43 a	33 a
H. virescens	36 a	40 a

[a] Percentages in the same column with letters in common are not significantly different according to a x^2 2x2 test of independence ($P < 0.05$), $n = 20$.

release bioassays in the wind tunnel (Table 5-4). There was no significant difference ($x^2 = 2.46$, $P > 0.1$) between the percent of BC_{15} and TBW males mated with H. virescens or BC_{15} females. Therefore, under these experimental conditions, competitiveness for mating of BC_{15} males was comparable to that observed in TBW males.

Backcross Male Sterility

When BC_{15} males were crossed or backcrossed with either conspecific or parental females, failure of the eggs to hatch confirmed the sterility of these males (Table 5-5). When BC_{15} males were crossed with H. virescens and BC_{15} females, the number of spermatophores per female (avg = 3.8, 4.1, respectively) was significantly different (Duncan's multiple range test, $P < 0.05$) from the number of spermatophores received by females crossed with H. virescens males (avg = 2.1). Similar results have been obtained by Karpenko and Proshold (1977) when they backcrossed BC^1 males with TBW females; the number of multiple matings was greater in these crosses than when females were paired with H. virescens males.

Laboratory studies by Pair et al. (1977) indicated that BC virgin females were competitive with H. virescens females for fertile matings with H. virescens or BC males. However, matings with BC males caused that BC or H. virescens females mated more often than females paired with TBW. Similar female mating behavior with BC insects has been reported by Proshold & LaChance (1974).

Table 5-5. Mean number of spermatophores and percent egg hatch when either H. subflexa, or H. virescens were paired with conspecific or BC_{15} adults.

Type of cross (♀ x ♂)	% females mated	Avg[a] no. spermatophores	Hatchability %
Hs x Hs	90	2.2 a	81
Hv x Hv	90	2.4 a	73
BC x BC	100	4.1 b	0
Hs x BC	100	2.1 a	0
Hv x BC	90	3.8 b	0
BC x Hv	100	2.3 a	80
BC x Hs	70	1.3 a	24

[a] Means followed by the same letter are not significantly different, Duncan's multiple-range test, $P < 0.05$ ($n = 10$).

The presence of spermatophore, sperm and/or related testicular fluids in the bursa copulatrix have been considered necessary to turn on the ovipositional response in most Lepidoptera (Bush et al. 1976, Thibout 1979). Usually, the onset of this ovipositional behavior is coupled with the cessation of calling behavior. In Pectinophora gossypiella (Saunders) irradiated males did not elicit as great an ovipositional response as control (unirradiated) males. P. gossypiella females with a spermatophore but only apyrene sperm oviposited few eggs (LaChance et al. 1978).

In Acrolepiopsis assectella Zell., the presence of eupyrene sperm in the spermatheca was necessary for oocyte production and increased oviposition (Thibout 1979). When more mobile spermatozoa were in the receptaculum seminalis, more oocyte production was observed, and more eggs were oviposited. In Manduca sexta (L.), mechanical stretching of the bursa by a sterile spermatophore caused only a temporary cessation of calling behavior while M. sexta females that a spermatophore and sperm from a normal male did not call again (Sasaki & Riddiford 1984).

Obviously, the set of mating changes the physiological status of the female. Depending on the species, the presence of a spermatophore, eupyrene spermatozoa and/or male fluids in the bursa, spermatheca or in the receptaculum seminalis would inhibit female receptivity for mating and turn on the ovipositional response.

Since BC males tend to mate repetitively with TBW and conspecific females, it was assumed that the females were receptive to mating more often and over a longer period than females crossed with normal males. Thus, the spermatophore produced by the BC males may not be sufficient to switch the calling behavior of TBW or BC female to an ovipositional response. If regulation of sexual behavior and reproductive activity in H. virescens and hybrid progeny originates in the presence of mobile eupyrene spermatozoa, it will be difficult to evaluate the effectiveness of releasing BC males (they have a limited amount of mobile eupyrene spermatozoa). In effect, females mated with these BC males will have a longer period of sexual receptivity and a reproductive potential similar to that of virgin females. If the local TBW population is low and the released ratio of BC males is high enough, the probability that a BC male-mated female (either BC or TBW) will mate with other BC males will be high enough to introduce the sterility factor in the native population. Conversely, when the natural population is high, females will mate more frequently with untreated native males and have normal progeny.

After the massive release of backcross insects on St. Croix, U.S. Virgin Islands to suppress the tobacco budworm Proshold (1983) concluded that BC-to-TBW ratios of 30:1 would be necessary to control H. virescens. These ratios possibly are required due to the repeated mating of females

which first mated with BC males. Evaluating numbers of mating and egg production from castrated parental males crossed with specific and backcross females might suggest some possibilities for a role of spermatophore, male fluids, and eupyrene spermatozoa on the regulation of reproductive activity in these species.

CHAPTER 6

INFLUENCE OF THE OVARIES AND TESTES ON THE COURTSHIP BEHAVIOR OF HELIOTHIS SUBFLEXA

Introduction

Female calling and male courtship behaviors are the principal components necessary for the achievement of mating in noctuid species (Haynes et al. 1983, Teal 1981). Usually, noctuid insects are short-lived as adults, laying their eggs and dying within a few days. Considering this natural history, Barth (1965) proposed that the endocrine system might not influence the regulation of mating behavior in these short-lived insects.

Hollander & Yin (1985) found that the corpora cardiaca, corpora allata and ovaries did not play any role in regulating either calling behavior or pheromone release in Lymantria dispar (L.). They suggested that possibly the brain regulated pheromone release. Further research by Raina & Klun (1984), Raina & Menn (1987), and Teal & Tumlinson (1989) has indicated that a peptide from the brain operates in the regulation of pheromone biosynthesis in Heliothis zea (Boddie).

In addition to neural structures, the gonads have been connected with mating behavior although the results are

variable. Barth & Lester (1973) reported that ovaries are important for the exhibition of calling behavior and pheromone release in the cockroach. On the other hand, ovariectomy in L. dispar did not interfere with either calling or pheromone release (Hollander & Yin 1985). Removal of the testes from pupae of Hyalophora cecropia (L.) did not prevent males from mating (Riddiford & Ashenhurst 1973).

Since castrated H. subflexa males may simulate the physiological status of backcross males (both are sterile), it would be interesting to determine the effect of removal of the testes on the male's ability to perform the courtship sequence. Therefore, in this Chapter the influence of H. subflexa gonads over calling behavior, pheromone release, and male courtship performance will be ascertained.

Materials and Methods

Microsurgery to remove the ovaries and testes was performed when the larvae were in last instar, two-three days before pupation. Two groups of larvae were immersed in tap water (10 - 20 min) for anaesthetization (Shirk, personal communication). The first group was sham operated and served as control, while larvae of the second group received full surgical treatment. Carefully, using surgical micro-scissors, two small longitudinal incisions (≈ 2 mm) were made on each side of dorsal vessel of the fifth abdominal segment of larvae. Watchmaker's forceps (# 5)

were used to grasp and pull the ovaries, one at time, to the incision where the connections with the surrounding tissue could be cut and the ovaries removed. The testes were located at both sides of the dorsal vessel proximally in the fifth segment. The dissection was performed in Weevers' saline solution (Weevers 1966), and the wound was sealed with Krazy Glue® a Cyanoacrylate ester. After the operation, the insects were placed on fresh pinto bean diet and kept under standard conditions. Four days later the larvae were checked and those presenting infection or a marked decrease in size were discarded. All surviving insects were observed for calling behavior and bioassayed for courtship behavior under wind tunnel conditions.

Results and Discussion

Mortality in control-operated and ovariectomized females was 11 and 20 percent, respectively. In males there was 6 and 11 percent mortality between the sham operated and orchidectomized groups. The higher mortality in females probably was due to the relative difficulty of extracting the small ovaries from the hemocoel. Individuals that survived showed only a diminutive dark scar on each side of the dorsal vessel or a small discolored area after pupation, and operated adults were externally indistinguishable from normal insects. Abdomens of sham operated females contained normal numbers of eggs, while the abdominal cavity of ovariectomized females contained no oocytes.

Removal of ovaries had no effect on calling behavior (Table 6-1). Females adopted the same calling posture described in Chapter 3, and they called in the same proportion that control females called ($x^2 = 2.63$, $P = 0.238$). The presence of a spermatophore in mated females as well as the absence of eggs in ovariectomized females was verified via dissection.

Orchidectomized males were able to respond to control and ovariectomized calling females during wind tunnel bioassays. These males performed the stereotypical courtship behavior observed in normal males and mated in the same proportion with control or ovariectomized females ($x^2 = 2.63$, $P = 0.10$) (Table 6-1). Moreover the stimulation of the male's normal courtship behavior sequence, under wind tunnel conditions, confirmed that calling by ovariectomized females was accompanied by pheromone release. Removal of the testes in males did not interfere with either their behavioral response to sex pheromone stimuli or copulating ability.

Results are similar to those of Hollander & Yin (1985). They found that ovariectomy of the last instar of L. dispar larva did not interfere with the development and morphology of the pheromone glands, calling behavior, and pheromone release, as measured by the female's capacity to arouse male-wing fanning. As with H. subflexa, L. dispar has a short adult life span and a single ovarian cycle.

Table 6-1. Effect of ovariectomy or orchidectomy on calling behavior and mating of H. subflexa.

Treatment	Percent[a] calling	mated with operated males
Control females	(16) 93	(13) 69
Operated females	(16) 87	(10) 80

[a] There was no significant difference in calling and mating between treatments groups (x^2 2 x 2 test of independence ($P < 0.05$). Values in parenthesis indicate n.

Castrated H. cecropia pupa formed normal adult male moths which mated and produced sterile but well formed spermatophores (Riddiford & Ashenhurst 1973). However, females mated with these sterile males failed to switch to an oviposition pattern. Sasaki & Riddiford (1984) reported that removal of testes in Manduca sexta did not prevent mating, but females mated with these males showed a transient cessation of calling (normal mated females did not call a second time), and had a much lower rate of oviposition. Results from this study show that removal of ovaries had no adverse effects in calling and in the release of pheromone or in male courtship performance (Table 6-1). The effect of these castrated males on the calling behavior of mated females as well as on ovipositional behavior was not evaluated. Therefore, it would be interesting to know if in the parental species the presence of a spermatophore in the bursa is sufficient to cause females to switch to oviposition behavior after mating with castrated males or if they reinitiate calling behavior as reported for M. sexta.

Failure of the mated female to stop calling and switch to an ovipositional behavior are important factors in the sexual behavior of backcross insects. If BC or TBW females mated with BC males are receptive longer than native females, the introduction of sterility in a natural population would be slowed, and it would require release of more insects over a longer period.

CHAPTER 7

CONCLUSIONS

Laster et al. (1972) showed that crosses between H. subflexa females and tobacco budworm (TBW), H. virescens, males produced sterile male progeny. Hybrid female descendants were fertile when they were backcrossed with H. virescens males. On the basis of these effects, Laster (1972) proposed that the release of hybrid males would be useful in applying the sterile male method to control H. virescens. In this context, an understanding of the mechanisms operative in sexual chemical communication among the species involved would serve, eventually, to predict behavioral interactions of these insects under field conditions.

The experiments described in Chapter 3 showed that the calling posture (and associated pheromone release) of H. subflexa, H. virescens, and backcross females had a circadian nature, because it persisted when the insects were maintained under continuous light intensity (ca. 1.2 lux). This endogenous calling periodicity in H. subflexa females occurs 4 to 6 h into the scotophase. The calling interval of backcross females was between 5 and 9 h after lights-off

with a peak period (> 60 percent calling females) from 6 to 8 hours. The timing of this daily BC female rhythm coincided with the calling period observed in TBW females. The early part of these interims overlapped the late portion of the H. subflexa calling period suggesting that the gland exposure period of BC and parental females were not sufficiently distinct to effect a great degree of reproductive isolation.

In the absence of females, searching behavior of BC and parental males recurred in circadian cycles corresponding to those observed in conspecific females. Thus, periodicity in mating would result from a rhythmic release of pheromone by females and a correlated response rhythm in males.

Wind tunnel studies indicated that the courtship behavior of H. subflexa was stereotyped and similar to the behavioral repertoire described for other noctuid moths (Ellis & Brimacombe 1980, Teal 1981, Raina et al. 1986). Analysis of a one step transition table giving frequencies of preceding and following acts in H. subflexa sexual behavior permitted identification of the main behaviors. They were categorized as follows: activation, random and oriented flight, landing, antennation, attempt copulation, and mating. Antennation appeared to be the behavioral step where a H. subflexa female recognized her mate.

Although there was a certain amount of flexibility in H. subflexa behavior, some males were able to mate

successfully without completing performance of all behaviors. These results support the hypothesis that the males which followed the stereotypical behavior had a greater probability of success in sexual reproduction. The final outcome of H. subflexa sexual encounters indicated that of 43 pairs tested, 27 (62 percent) mated successfully.

Mating experiments with BC_6 insects were characterized by a marked low flight activity within the male population. Courtship behaviors of BC_6 were essentially the same as those described for H. virescens moths (Teal 1981). The BC_6 males did not antennate the female ovipositor, but they displayed profusely their well developed hairpencils as did TBW males stimulating the female to assume a receptive posture for copulation. This close-range behavior probably was the step in the mating sequence necessary for mutual recognition. Absence of hairpencil display and associated male pheromone (Teal et al. 1986) may allow BC or TBW female to escape when it is courted by other moth species (e.g., H. subflexa males). Overall results indicated that of 51 reproductive encounters between BC_6 males and BC_6 females, only 17 (33 percent) culminated in mating.

An acquiescent female, antennation (in H. subflexa) or full hairpencil display (in BC and TBW males), accurate parallel movement, and the male's ability to clasp the female's genitalia were identified as requisites for successful mating for these insects. Failure of the male to

exhibit a precise parallel movement and a proper clasping of the female's genitalia were the primary factors that increased the probability of an unsuccessful mating. The backcross male's tendency to settle down after a brief initial activity contributed greatly to an increased proportion of unsuccessful males.

Trapping studies (Teal 1981) revealed that close-range behaviors (e.g., landing) in H. subflexa males were not elicited by the reported synthetic sex pheromone. Hence, an analysis of the relationship between pheromonal chemicals and evoked behaviors (Chapter 4) was initiated.

Analyses of gland tips indicated that the mean percent composition of H. subflexa sex pheromone was: 16:Al, 0.98; Z9-16:Al, 25; Z11-16:Al, 60; Z7-16:Ac, 0.3; Z9-16:Ac, 2.5; Z11-16:Ac, 5.7; Z9-16:OH, 0.5; Z11-16:OH, 4. Total extracted pheromone from female ovipositor tips (n = 15) was 201.4 ± 12.5 ng/female.

Male behaviors in response to crude extracts of females ovipositors applied to filter paper as identified using wind tunnel studies were random flight, oriented flight, contact, and landing. Data analysis indicated that H. subflexa male behavior durations elicited by these extracts were similar to those observed using calling virgin females in cages. In both bioassays, the males did not attempt copulation with the source, suggesting that visual or tactile stimulation may contribute to this close-range behavior.

Deletion of Z9-16:Al or Z11-16:Al from the chemical blend prevented males from displaying any signal of excitation ($P < 0.05$). During the bioassays of these deleted synthetic blends, nearly all of the male moths flew randomly. Therefore, the main aldehydes (Z9-16:Al and Z11-16:Al) are essential in H. subflexa's sexual signaling system.

Similar to H. subflexa Z11-16:Al is also the main component in the pheromone of H. zea, H. armigera, and H. virescens, and H. phloxiphaga (Tamaki 1985, Raina et al. 1986), and hybrids (Klun et al. 1982, this study). Published data (Kehat et al. 1980, Teal 1981, Klun et al. 1982, Raina et al. 1986, Teal et al. 1986) and results from this study suggest that Z11-16:Al may operate with other minor pheromonal components in inducing male attraction in the early phases of the courtship behavior of Heliothis species.

Pheromone blends which lacked 16:Al or Z9-16:OH were not significantly different in timed behavioral response or the percent of males landing compared to the full 7 component blend. The function of hexadecanal is variable for the various Heliothis species. Omission of this compound from the H. virescens pheromonal blend caused a significant decrease in close-range (landing, hairpencil display) behavior of males (Teal et al. 1986). Exclusion of 16:Al from H. phloxiphaga's sex pheromone mixture did not

suggest any obvious behavior-inducing role for this compound (Raina et al. 1986).

Subtraction of individual acetates from the pheromonal synthetic blend caused a significant decrease in the percent of males landing on the dispenser and in the time that male moths remained on the source. These compounds probably induce subtle effects in close-range behaviors (landing, stay on the source). Also, they may contribute to the maintenance of species isolation because, to date, no other Heliothis moths include acetates in their sex pheromone. Elimination of Z11-16:OH resulted in a significant decrease ($P < 0.001$) in the percent of H. subflexa males landing as well as a decrease in the time that the moth stayed on source. Thus, this alcohol in conjunction with acetates maximizes the signals in the chemical background for inducing landing by H. subflexa males and stimulation of a subsequent search on the source.

Field experiments confirmed predictions from wind tunnel studies. The presence of Z11-16:OH in the pheromone bait was a decisive factor in inducing H. subflexa males to land in sticky or bucket traps. Deletion of Z7-16:Ac and Z9-16:OH from the synthetic blend did not result a reduction in trap catch when compared with catches from traps baited with two virgin females.

Field trials permitted definition of the proper release ratio of Z11-16:OH from rubber septa dispensers. Male

captures in traps without this alcohol in the bait dispensers were significantly lower than traps with the full mixture. A release ratio of two percent of Z11-16:OH was optimal for catching males in sticky or bucket traps. A release ratio of Z11-16:OH higher than five percent of the total blend decreased the number of males trapped. Inclusion of Z11-16:OH in the pheromonal blend of H. zea, H. virescens, and H. armigera caused inhibitory responses in the respective males (Teal 1984, Teal et al. 1986). In H. phloxiphaga (Raina et al. 1986) and H. subflexa (this study) Z11-16:OH facilitated close-range behaviors.

For trapping studies, a precise release ratio and dosage of sex pheromone components are required to obtain male (or females) captures similar to those evoked by the opposite sex. To date formulations effective in achieving this goal have been very limited (Baker et al. 1980, Buttler & McDonough 1981, Heath et al. 1986, Cardé 1988). In this study, it was possible to predict the release ratio of pheromone from rubber septa dispensers using the techniques of Heath et al. (1986). Therefore, different pheromone blends and release ratios should be screened using a volatile collection system before synthetic lures are used under field conditions.

Besides the behavioral repertoire, the BC chemical pheromone components (Chapter 5) were identical to those found in H. virescens. Analyses of the BC sex pheromone

gland showed the presence of the following compounds: tetradecanal, (2.1%); (Z)-9-tetradecenal, (1.7%); hexadecanal, (8.1%); (Z)-7-hexadecenal/(Z)-9-hexadecenal, (2.7%); (Z)-11-hexadecenal, (79.2%); and (Z)-11-hexadecen-1-ol (4.5%). The ratios of (Z)-9-hexadecenal:(Z)-11-hexadecanal in females species were: 1:39 in BC, 1:5.6 in hybrids, 1:2.6 in H. subflexa, and 1:43 in H. virescens.

Male behaviors evoked by crude extracts loaded onto filter paper included: oriented flight, landing, hairpencil display, and attempt copulation. There were no significant differences in the landing times of H. virescens elicited by pheromone extracts from conspecific and backcross females. Therefore, chemicals from BC females were as competitive for attracting TBW males as chemicals from TBW females. Landing response in BC and TBW males was not elicited by crude pheromone extract from H. subflexa females.

Comparison of the response of BC_4, BC_8, and BC_{12} to TBW pheromone extract revealed that males of the early generations (i.e., BC_4) remained on the source significantly less time than males from the later backcrosses (BC_8 and BC_{12}) or TBW males. These results and data from the mating behavior studies, suggest that initial backcross males may not be as competitive as males from later BC generations.

Although the results here indicate that early BC females (BC_1-BC_4) appear to be competitive for mating with conspecific or TBW males, previous studies at Stonesville,

MS (Laster et al. 1978) indicated that BC females are fully competitive for mating by the BC_3 generation. Also, Laster et al. (1988) reported that although the average number of eggs and percent hatch were low for the first two backcrosses, these numbers increased in successive BC generations. Thus, the use of later female generations (> BC_6) would be advisable in field experiments.

At this time, it is not possible to distinguish if the differential response between early and later BC males to crude extracts is caused by genetic anomalies present in the first BC male generations, by a founder effect, and/or rearing conditions. However, data from this study suggest that later BC generations (> BC_6) would be more competitive in sexual encounters with conspecific or heterospecific males than their early counterparts.

Published data (Karpenko & Proshold 1977, Pair et al. 1977, Martin et al. 1984) and results from this study (Chapter 6), suggest that BC and TBW females mated with BC males were receptive to mating longer than females crossed with parental males. It appears reasonable to assume, therefore, that the spermatophore produced by BC males may not be sufficient to switch the calling behavior of TBW or BC to an ovipositional response as reported in other insect sterility studies (LaChance et al. 1978, Thibout 1979, Sasaki & Riddiford 1984).

Ovariectomies performed on H. subflexa pupae (Chapter 6) had no adverse effects on the calling behavior of adult females. Observation of the courtship behavior of H. subflexa males under wind tunnel conditions confirmed that calling by females without ovaries was accompanied by pheromone release. Removal of the testes from H. subflexa males did not interfere with the male's behavioral response to sex pheromone or mating capacity. Post-mating female behavior was not evaluated.

Additional experiments with castrated H. subflexa and H. virescens males crossed with conspecific and BC females may suggest possibilities on the role (if any) of spermatophore, male fluids, and eupyrene spermatozoa on the regulation of post-mating activity in these insects. It will be interesting to determine if females (either BC or TBW) mated with sterile parental males require more matings that females mated with normal males as suggested by this study.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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